

Development of enzyme technology for modification of functional properties of xylan biopolymers

by

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part, submitted it at any university for a degree.

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Summary

There is growing interest to utilise xylan as speciality biopolymers in similar ways as high molecular weight polysaccharides such as starch and cellulose. The need to utilise xylan as alternative to cellulose and starch has increased because the cellulose and starch have many other competing uses. Unlike cellulose and starch, xylans are heteropolymers with higher degree of substitution and are of lower molecular mass and therefore, do not readily become insoluble to form hydrogels and biofilms. Consequently, xylans do not suit applications of starch and cellulose as speciality biodegradable additives and coatings in the food, pharmaceutical, pulp and paper and textile and many other industries. This study was conducted to develop an enzyme technology, based on recombinant α -L-arabinofuranosidase and purified α -D-glucuronidase with polymeric xylan substrate specificity, for controlled reduction of the solubility of water soluble polymeric xylan, leading to formation of insoluble nanohydrogels.

Although xylan is available in abundance, a large proportion of it is currently wasted in lignocellulose process waste streams with little prospects for recovery and addition of value. Lignocellulosic materials including *Eucalyptus grandis*, *Pinus patula*, *Bambusa balcooa* (bamboo) and sugarcane (*Saccharum officinarum* L) bagasse (bagasse) found in South Africa were investigated as sources of water soluble xylan for enzyme modification. Two mild alkali-low temperature methods (alkali charge of < 14% and temperature of < 80°C), one with ultrapurification denoted as the Hoiye and the other with ethanol precipitation, denoted as Lopez method, were evaluated for their selective extraction of water soluble xylans from the specified lignocellulosic materials. The water soluble xylans were extracted from *P. patula*, bagasse, *E. grandis* and bamboo by the Hoiye method with extraction efficiencies of 71.0, 66.0, 35.0 and 20.0% respectively. Using the Lopez method, the xylans from bagasse and *E. grandis* were extracted with extraction efficiencies of 28.0 and 12.0% respectively. The xylans extracted from *P. patula*, bamboo and bagasse were identified as arabinoglucuronoxylans, which were substituted with arabinose and 4-O-methyl-D-

glucuronic acid (MeGlcA) side chains, whereas, the xylan extracted from *E. grandis* were identified as 4-*O*-methyl- β -D-glucuronoxylan (glucuronoxylan) substituted with MeGlcA groups on the main xylan chain. In addition, the glucuronoxylans contained some traces of arabinose and rhaminose sugar residues. The extracted xylan fractions had degree of polymerisation (DP) of > 10 and were water soluble, which suited the required properties of xylans for customised enzyme modification.

The selective removal of the arabinose, MeGlcA and acetyl groups to create linear regions of xylose units in xylans that causes intra and inter-polymer bonding is considered to be the key process for reducing the solubility of water soluble xylans. The α -L-arabinofuranosidase of *Aspergillus niger* (AbfB) and α -D-glucuronidase of *Schizophyllum commune* (AguA) are special enzymes so far identified with the ability to selectively remove arabinose and MeGlcA side chains respectively, from water soluble xylans. Large scale application of the AbfB and AguA for reducing solubility of the water soluble xylans would require their extracellular production in large quantities and free of contamination from the xylan main chain degrading enzymes including the endo-1,4- β -xylanase. Selective production of the AbfB free of xylanase activity was achieved in recombinant *A. niger* D15 [*abfB*] strain under the transcriptional control of the glyceraldehyde-3-phosphate dehydrogenase promoter (*gpd_P*) and glucoamylase terminator (*gla_T*). The recombinant AbfB was secreted extracellularly in 125 mL shake flasks and 10 L bioreactor fermentation cultures with volumetric activities of up to 10.0 and 8.0 nkat mL⁻¹ respectively, against *para*-nitrophenol arabinofuranoside (*pNPA*). The secretion of the recombinant AbfB was growth associated and therefore, increased up to 2.5 times with addition of concentrate corn steep liquor (CCSL) as an additional source of nitrogen in the 2 x minimal standard cultivation media. The biomass specific activity of the recombinant AbfB against the *pNPA* substrate was approximately 366 nkat g⁻¹ (dry weight basis). The recombinant AbfB displayed a single pure species band on 10% SDS-PAGE stained with Coomassie blue and had an estimated molecular mass of 67 kDa. In addition, the recombinant AbfB showed optimal activity at 40-55°C and pH 3.0-5.0 and was stable under cultivation, storage and operating conditions at temperatures between 30-60°C and pH 3.0-6.0. Furthermore, the recombinant AbfB showed broad substrate specificity selectively removing arabinose side groups from low viscosity wheat and oat spelt arabinoxylans, larchwood arabinogalactan, debranched arabinan

and araboglucuronoxylans extracted from bagasse, bamboo and *P. patula* found in South Africa,.

The recombinant AbfB was able to precipitate xylans extracted from bagasse, bamboo and oat spelt but not from *P. patula*. Over 95% of the activity of the recombinant AbfB against the *p*NPA was recyclable after selective hydrolysis of the xylan at 40°C for 16 h. On the other hand, the purified AguA enzyme could only precipitate the birch glucuronoxylan but not the glucuronoxylan extracted from *E. grandis* and arabinoglucuronoxylans extracted from bagasse, bamboo and *P. patula*. The synergetic action of the recombinant AbfB and the purified AguA increased the removal of the arabinose side chains from bagasse xylan by 22% and from bamboo xylan by 33%, whereas, the removal of the MeGlcA side chains from bagasse xylan increased by only 5% and that from bamboo xylan decreased by 13%. The selective removal of the arabinose side chains from oat spelt, bagasse and bamboo xylans by the recombinant AbfB had higher apparent viscosity relative the corresponding untreated xylans. However, the apparent viscosity of both the treated and untreated xylans reduced with increased shear rate. The viscosity had an overall negative correlation with arabinose side chain removal reaching a minimum of 2.03 mPa.s for hydrolysis of oat spelt xylan that was performed for 9.0 h at a temperature of 45.8°C with recombinant AbfB xylan specific dosage of 400.0 nkat g⁻¹_{substrate}. The alteration of the viscosity of the xylans by the selective removal of the side chains is of special interest in the production of speciality emulsifying, thickening and antifoaming agents.

The optimal values for hydrolysis time, enzyme dosage and temperature for maximum degree of removal of arabinose side chains from oat spelt xylan by the recombinant AbfB and of the removal of MeGlcA side chains from birch xylan by the purified AguA were determined by the Box-Benhken response surface method (RSM). The experimental region covered the xylan specific dosage for the recombinant AbfB between 18.0 and 540.0 nkatg⁻¹_{substrate} and for the purified AguA xylan between 2.0 and 18.0 µkatg⁻¹_{substrate} at temperatures between 30 and 50°C and hydrolysis time between 1 and 16 h. The temperature, enzyme xylan specific dosage and hydrolysis time had significant effect ($p < 0.05$) on both the selective removal of arabinose from oat spelt xylan by the recombinant AbfB and the selective removal of MeGlcA from

birch xylan by the purified AguA. However, the interaction of these hydrolysis parameters were significant ($p < 0.05$) on only the removal of arabinose side chains from oat spelt xylan by the recombinant AbfB. The optimal values for hydrolysis time, temperature and xylan specific dosage were estimated to be 14-16 h, 38-45°C and 607.0 nkatg⁻¹_{substrate} respectively, for maximum removal of 43% of the available arabinose in oat spelt xylan by the recombinant AbfB. Whereas, the optimal values for hydrolysis time, temperature and xylan specific dosage for maximum removal of 0.5% of the available MeGlcA side chains from the birch xylan by the purified AguA were estimated to be 11 h, 38°C and 18.0 µkatg⁻¹_{substrate} respectively. The optimal values of the hydrolysis parameters for both the removal of the arabinose from oat spelt xylan by the recombinant AbfB and of MeGlcA side chains from birch by the purified AguA could be predicted using quadratic models that fitted the response surface plots with regression coefficients of > 0.9.

The effects of *in situ* selective removal of arabinose and MeGlcA side chains by AbfB and AguA respectively, from water soluble xylans, on their precipitation and adsorption onto cotton lint were investigated. The cotton lint was treated with xylans extracted from bagasse, bamboo, *P. patula* and *E. grandis* using the Hoiye method in the presence of the recombinant AbfB, AguA and the cocktail of the two enzymes. The effects of *in situ* selective hydrolysis of model xylans including birch, oat spelt and H₂O₂ bleached bagasse and *E. grandis* xylan gel by the enzymes on their adsorption onto cotton lint were used for reference purposes. The purified AguA increased the adsorption of arabinoglucuronoxylans extracted from bagasse bamboo and *P. Patula* using the Hoiye method onto cotton lint the most compared to the effect of the recombinant AbfB and the cocktail of the recombinant AbfB and purified AguA. The purified AguA increased the adsorption of the xylans extracted from bagasse and *E. grandis* xylans by 334 and 29% respectively, but decreased that of *E. grandis* xylan gel and H₂O₂ bleached bagasse xylan by 31 and 6% respectively. Similarly, the presence of the recombinant AbfB increased the adsorption of the bamboo, *P. Patula* and oat spelt xylans by 31, 44 and 900% respectively, but decreased the adsorption of the xylan extracted from bagasse and the H₂O₂ bleached bagasse xylan by 13 and 30% respectively. Furthermore, different xylan-cellulose interactions and water adsorption capacities of the cotton lint were observed with the *in situ* modification and adsorption of the xylans extracted from bagasse, bamboo, *E.*

grandis and *P. patula* in the presence of the recombinant AbfB and purified AguA. Therefore, the enzyme aided adsorption of xylans could be used to alter or improve functional properties of cellulosic materials.

The performance of enzymatically formed xylan nanohydrogels as encapsulation matrices for slow delivery of bioactive agents was evaluated. Insoluble xylan nanohydrogels formed by selective removal of arabinose side chains from water soluble oat spelt xylan by the recombinant AbfB were characterized for particle size distribution, surface charge (zeta potential), morphology stability and ability to encapsulate and slowly release the HRP. The enzymatically formed oat spelt xylan hydrogels were spherical in shape with particle sizes ranging from 18 nm to > 10 000 nm. The xylan nanohydrogels exhibited a negative zeta potential of up to -19 mV and displayed self assembling behaviour when formed at xylan concentrations of higher than 1.5% (w/v) and hydrolysis time beyond 17 h. The xylan concentration significantly ($P < 0.05$) influenced both the particle size and zeta potential of the oat spelt xylan nanohydrogels whereas the recombinant AbfB hydrolysis time was significant ($P < 0.05$) on the zeta potential. The oat spelt xylan nanohydrogels successfully encapsulated the HRP enzyme both during and after formation of the oat spelt xylan nanohydrogels and the release of the encapsulated HRP in active form, was sustained for a period of 180 min. Therefore, the xylan side chain removing enzymes have a role in preparation of biodegradable nanoencapsulation devices.

Overall, the AbfB and AguA have presented a novel tool for functionalising water soluble xylans to be used as speciality additives, coating and implantation or encapsulation matrices, with reduced impact on the environment. This will advance processing and expand the product spectrum of lignocellulosic materials.

Opsomming

Daar is 'n toenemende belangstelling om spesialiteit biopolimere uit xilaan ontwikkel, en op soortgelyke wyse as hoë molekulêre massa polisakkariede soos stysel en sellulose te benut. Die behoefte om xilaan biodegradeerbare polimere as 'n alternatief tot sellulose en stysel te gebruik neem toe omdat laasgenoemde baie ander kompeterende gebruike het. Anders as sellulose en stysel is uit xilaan heteropolimere met 'n hoë graad van substitusie in die hoofketting met sygroepe en lae molekulêre massas, en raak daarom nie geredelik onoplosbaar om hidrojel en biofilms te vorm nie. Gevolglik is xilaan nie geskik vir toepassings van stysel en sellulose as spesialiteit biodegradeerbare bymiddels en bedekkings in die voedsel-, farmaseutiese-, pulp en papier-, tekstiel-, en vele ander industrieë nie. Hierdie studie is uitgevoer om 'n ensiemtegnologie te ontwikkel gebaseer op rekombinante α -L-arabinofuranosidase en gesuiwerde α -D-glukuronidase met polimeriese xilaan substraat spesifisiteit, vir beheerde vermindering van die oplosbaarheid van wateroplosbare polimeriese xilaan wat lei tot die vorming van onoplosbare nanohidrojels.

Alhoewel xilaan volop beskikbaar is, word 'n groot deel daarvan tans vermors in afvalstrome uit lignosellulose prosessering, primêr verpulping, met min vooruitsigte vir herwinning en toevoeging van waarde. Lignosellulose materiaal wat in Suid-Afrika geproduseer word, insluitend *Eucalyptus grandis* (*E. grandis*), *Pinus patula* (*P. patula*), *Bambusa balcooa* (bamboes) en suikerriet (*Saccharum officinarum* L) (bagasse), is ondersoek as bronne van wateroplosbare xilaan vir ensiem modifikasie. Twee gematigde, lae temperatuur alkali-metodes ('nalkali lading van < 14% en temperatuur van < 80°C), een met ultrasuiwering aangedui as Hoije en die ander met etanolpresipitasie aangedui as Lopez metode, is evalueer vir selektiewe ekstraksie van wateroplosbare xilaan vanuit die genoemde lignosellulose materiale. Die wateroplosbare xilaan is ge-ekshaheer vanuit *P. patula*, bagasse, *E. grandis* en bamboes met die Hoije metode met ekstraksie doeltreffendhede van 71.0, 66.0, 35.0, en 20.0%, onderskeidelik. Met die Lopez metode is xilaan vanuit bagasse en *E. grandis* geëkstraheer met ekstraksie doeltreffendhede van 28.0% en 12.0%, onderskeidelik. Die xilaan wat vanuit *P. patula*, bamboes, en bagasse ekstraheer is, is as arabinoglukuronoxilaan geïdentifiseer, wat met arabinose en 4-O-metiel-D

glukuronsuur sykettings vervang is, terwyl die xilaan wat vanuit *E. grandis* ekstraheer is as 4-*O*-metiel- β -D-glukuronoxilaan (glukuronoxilaan), met substitusie met MeGlcA en asetiel-groepe op die hoof xilaan-ketting (ruggraat) is. Die glukuronoxilaan het verder spore van arabinose en rhaminose funksionele groepe bevat. Die geëkstraheerde xilaan fraksies het grade van polimerisasie > 10 gehad en was wateroplosbaar, wat die vereiste eienskappe van die xilaan vir doeltreffende ensiem modifikasies bevredig het.

Die selektiewe verwydering van die arabinose, MeGlcA, en asetiel-groepe om xilose eenhede sonder substitusie in polimeriese xilaan te vorm, wat intra- en inter-polimeer binding veroorsaak, word beskou as die belangrikste proses vir die vermindering van die oplosbaarheid van wateroplosbare xilaan. Die α -L-arabinofuranosidase van *Aspergillus niger* (AbfB) en α -D-glukuronidase van *Schizophyllum commune* (AguA) is spesialiteitsensieme wat tot dusver is met die vermoë om selektief die arabinose en MeGlcA sykettings, onderskeidelik, vanaf wateroplosbare xilaan te verwyder. Groot skaalse toepassing van die AbfB en AguA ensieme, vir die vermindering van die oplosbaarheid van wateroplosbare xilaan, sal ekstrasellulêre produksie deur mikrobies in groot hoeveelhede en vry van kontaminasie van die xilaan hoofketting degraderende ensieme insluitend die endo-1,4- β -xilanase vereis. Selektiewe produksie van die AbfB vry van xilanase aktiwiteit is verkry deur kultivering van rekombinante *A. niger* D15 [*abfB*], met transkripsie van die *abfB*-geen beheer deur die gliseraldehyd-3-fosfaat dehidrogenase promotor (*gpd*) en glukoamilase termineerder (*glaA*_T). Die rekombinante AbfB ensiem is ekstrasellulêr geproduseer in 125 mL skudflesse en 'n 10 L bioreaktor fermentasiekulture met volumetriese aktiwiteite van tot 10.0 en 8.0 nkat mL⁻¹, onderskeidelik, teen *para*-nitrofenol arabinofuranosied (*pNPA*). Die uitskeiding van die rekombinante AbfB was groei geassosieerd en het daarom tot 2.5 keer toegeneem met die byvoeging van gekonsentreerde meliweekvloeistof as 'n addisionele bron van stikstof in die 2 x minimale standaard kwekingsmedium. Die biomassa spesifieke aktiwiteit van die rekombinante AbfB teen die *pNPA* substraat was ongeveer 366 nkat g⁻¹ (droë massa basis). Die rekombinante AbfB het 'n enkele suiwer spesie band getoon op 10% SDS-PAGE gelek met Coomassie blou en het 'n beraamde molekulêre massa van 67 kDa gehad. Die rekombinante AbfB het verder optimale aktiwiteit by 40-55°C en pH 3.0-5.0 getoon

en was stabiel onder kweking-, storing-, en bedryfstoeestande by temperature tussen 30-60°C en pH 3.0-6.0. Die rekombinante AbfB het ook wye substraatspesifisiteit getoon om arabinose sy-groepe selektief te verwyder vanaf lae viskositeit koring-en hawerbiopolimere, lariks arabinogalaktaan, onvertakte arabinaan, en arabinoglukuronoxilaan biopolimere, geëkstraheer vanaf bagasse, bamboes en *P.patula* wat in Suid-Afrika aangetief word.

Die rekombinante AbfB kon xilaan, ge-ekshaheer vanaf bagasse, bamboes en hawer onoplosbaar maak, maar die xilaan geëkstraheer vanaf *P. patula* nie. Meer as 95% van die aktiwiteit van die rekombinante AbfB teen die *p*NPA kon hersirkuleer word na selektiewe hidrolise van die xilaan by 40°C vir 16 h. Aan die ander kant kon die gesuiwerde AguA-ensiem slegs berkehout glukuronoxilaan onoplosbaar maak, maar nie glukuronoxilaan wat vanaf *E. grandis* geëkstraheer is of arabinoglukuronoxilaan wat vanaf bagasse, bamboes en *P. patula* geëkstraheer is nie. Die sinergistiese aksie van die rekombinante AbfB en die gesuiwerde AguA het die verwydering van die arabinose sykettings vanaf bagassexilaan met 22% vermeerder en met 33% in die geval van bamboesxilaan. Die verwydering van MeGlcA sykettings vanaf bagassexilaan is met slegs 5% vermeerder, terwyl dit met 13% verminder het in die geval van bamboesxilaan. Die selektiewe verwydering van die arabinose sykettings vanaf xilaan van hawer, bagasse, en bamboes deur die rekombinante AbfB het hoër skynbare viskositeit gehad relatief tot die ooreenstemmende onbehandelde xilaan . Die skynbare viskositeit van beide die behandelde en onbehandelde xilaan het egter verminder met toenemende skuiftempo. Die viskositeit het 'n algehele negatiewe korrelasie met arabinose syketting verwydering gehad en het 'n minimum van 2.03 mPa.s bereik vir hidrolise van hawerxilaan wat uitgevoer is vir 9.0 h by 'n temperatuur van 45.8°C met rekombinante AbfB xilaan met 'n spesifieke dosering van 400.0 nkat g⁻¹_{substraat}. Die wysiging van die viskositeit van die xilaan deur die selektiewe verwydering van die sykettings is van besondere belang in die produksie van spesialiteit emulsifisering, verdikking- en skuimweermiddels.

Die optimale waardes vir hidrolisetyd, ensiendosering en temperatuur vir maksimum graad van arabinose syketting verwydering vanaf hawerxilaan met die rekombinante AbfB, en van MeGlcA syketting verwydering vanaf berkehout xilaan met die gesuiwerde AguA, is vasgestel deur middel van die Box-Benhken responsie-

oppervlak metode. Die eksperimentele gebied het die xilaanspesifieke dosering met die rekombinante AbfB tussen 18.0 en 540.0 nkat $\text{g}^{-1}_{\text{substraat}}$ en vir die gesuiwerde AguA xilaan tussen 2.0 en 18.0 $\mu\text{kat g}^{-1}_{\text{substraat}}$ by temperature tussen 30 en 50°C en hidrolisetye tussen 1 en 16 h gedek. Die temperatuur, ensiem xilaan spesifieke dosering en hidrolise tyd het elk 'n beduidende invloed ($p < 0.05$) gehad op beide die selektiewe verwydering van arabinose vanaf hawerxilaan met die rekombinante AbfB en die selektiewe verwydering van MeGlcA vanaf berkehout xilaan met die gesuiwerde AguA. Die interaksie van hierdie hidroliseparameters was egter net beduidend ($p < 0.05$) in die geval van arabinose syketting verwydering vanaf hawer xilaan met die rekombinante AbfB. Die optimale waardes vir die hidrolise tyd, temperatuur, en xilaan spesifieke dosering is beraam om gelyk aan 14-16 h, 38-45°C, en 607.0 nkat $\text{g}^{-1}_{\text{substraat}}$, onderskeidelik, te wees vir maksimale verwydering van 43% van die beskikbare arabinose in die hawer xilaan met die rekombinante AbfB. Die optimale waardes vir die hidrolise tyd, temperatuur en xilaan spesifieke dosering vir maksimale verwydering van 0.5% van die beskikbare MeGlcA sykettings vanaf die berkehout xilaan met die gesuiwerde AguA is beraam om gelyk aan 11 h, 38°C, en 18.0 $\mu\text{kat g}^{-1}_{\text{substraat}}$, onderskeidelik, te wees. Die optimale waardes van die hidrolise parameters, vir beide die verwydering van die arabinose vanaf hawer xilaan met die rekombinante AbfB en van MeGlcA sykettings vanaf berkehout met die gesuiwerde AguA, kon voorspel word deur gebruik te maak van kwadratiese modelle wat die responsie-oppervlak grafieke met regressie koëffisiënte > 0.9 gepas het.

Die effek van *in situ* selektiewe verwydering van arabinose en MeGlcA sykettings met rekombinante AbfB en gesuiwerde AguA, onderskeidelik, vanaf wateroplosbare xilaan op hulle presipitasie en adsorpsie op katoen lint is ondersoek. Die katoenlint is behandel met xilaan ge-ekstraheer vanuit bagasse, bamboes, *P. patula*, en *E. grandis* deur gebruik te maak van die Hoije metode in die teenwoordigheid van die rekombinante AbfB, AguA, en 'n mengsel van die twee ensieme. Die effek van *in situ* selektiewe hidrolise, deur die ensieme van model xilaan insluitende berkehout, hawer en H₂O₂-gebleikte bagasse en *E. grandis* xilaan jel, op hulle adsorpsie op katoen lint is gebruik vir verwysingsdoeleindes. Die gesuiwerde AguA het die adsorpsie van arabinoglukuronoxilaan, wat vanuit bagasse, bamboes en *P. patula* ekstraheer is deur middel van die Hoije metode, op katoenlint die meeste laat toeneem in vergelyking met die effek van die rekombinante AbfB en die mengsel van die rekombinante AbfB

en die gesuiwerde AguA. Die gesuiwerde AguA het die adsorpsie van die xilaan wat vanuit bagasse en *E. grandis* ekstraheer is met 334 en 29%, onderskeidelik, laat toeneem, maar het die adsorpsie van *E. grandis* xilaan en H₂O₂ gebleikte bagasse xilaan met 31 en 6%, onderskeidelik, laat afneem. Op 'n soortgelyke wyse het die teenwoordigheid van die rekombinante AbfB die adsorpsie van die bamboes, *P. Patula* en hawer xilaan met 31, 44, en 900%, onderskeidelik, laat toeneem, maar die adsorpsie van die xilaan ekstraheer vanuit bagasse en die H₂O₂ gebleikte bagasse xilaan met 13 en 30%, onderskeidelik, laat afneem. Verskillende xilaan-sellulose interaksies en water adsorpsie kapasiteite van die katoen lint is opgemerk met die *in situ* modifikasie en adsorpsie van die xilaan ekstraheer vanuit die bagasse, bamboes, *E. grandis* en *P. patula* in die teenwoordigheid van die rekombinante AbfB en gesuiwerde AguA. Die ensiem bygestaande adsorpsie van xilaan kon daarom gebruik word om die funksionele eienskappe van die sellulose materiaal aan te pas of te verbeter.

Die wekverrigting van ensimaties gevormde xilaan nanohidrojels as enkapsuleringmatrikse vir stadige vrystelling van bioaktiewe middels is geëvalueer. Onoplosbare xilaan nanohidrojels wat gevorm is deur selektiewe verwydering van arabinose sykettings vanaf wateroplosbare hawer xilaan met die rekombinante AfbA, is gekarakteriseer vir partikelgrootteverspreiding, oppervlaklading (zeta potensiaal), morfologiese stabiliteit, en die vermoë om die ramenase peroksidase te enkapsuleer en stadig vry te stel. Die ensimaties gevormde hawer xilaan hidrojels het 'n sferiese vorm gehad met partikelgroottes wat gewissel het van 18 nm tot > 10 000 nm. Die xilaan nanohidrojels het 'n negatiewe zeta potensiaal van tot -19 mV getoon, en het self-vormings gedrag vir partikels ten toon gestel indien dit by xilaankonsentrasies hoër as 1.5% (m/v) en hidrolise tye langer as 17 h gevorm is. Die xilaan konsentrasie het beide die partikelgrootte en die zeta potensiaal van die hawerxilaan nanohidrojels beduidend ($P < 0.05$) beïnvloed terwyl die rekombinante AbfB hidrolise tyd beduidend ($P < 0.05$) was op die zeta potensiaal. Die hawer xilaan nanohidrojels, het die ramenaseperoksidase ensiem suksesvol enkapsuleer, beide gedurende en na die vorming van die hawer xilaan nanohidrojels en die vrystelling van die geënkapsuleerde ramenase peroksidase in aktiewe vorm is volgehou vir 'n periode van 180 min. Die ensieme wat die syketting van die xilaan verwyder het, het dus 'n rol in die voorbereiding van biodegradeerbare nano-enkapsulasie geëdskap.

In die geheel veskaf die rekombinante AbfB en gesuiwerde AguA 'n nuwe stel manier voor om wateroplosbare xilaan te funksionaliseer om as spesialiteit bymiddels, bedekking, en inplanting of enkapsulasiematrikse gebruik te word met 'n verminderde impak op die omgewing. Dit sal prosessering bevorder en die produkspektrum van lignosellulose materiale uitbrei.

Dedication

Dedicated to my beloved parents, Mrs Esther Jelenje and Late father, Mr. Abel Yakobe Jelenje, whose wisdom continues to inspire me.

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Abbreviations

1. ANOVA	Analysis of variance
2. ASTM	American Standard Measurement
3. ATCC	American type culture collection
4. COD	Chemical oxygen demand
5. dH ₂ O	De-ionised water
6. DME	Department of Minerals and Energy
7. DNS	Dinitrosalicylic acid
8. DO	Dissolved oxygen
9. DP	Degree of polymerisation
10. DS	Degree of substitution
11. DW	Dry weight
12. ED	Electrochemical detector
13. FDA	Food and Drug Administration
14. FTIR	Fourier transmission infrared
15. GlcA	Glucuronic acid
16. GRAS	Generally regarded as safe
17. HPAEC-PAD	High performance anionic exchange with pulsed amperometric detector
18. ¹ H-NMR	Proton nuclear magnetic resonance
19. HRP	Horse radish peroxidase
20. kDa	kilo Dalton
21. K _m	Michael-Menten's constant
22. LAP	Laboratory analytical procedure
23. LCC	Lignin carbohydrate complex
24. MeGlcA	4- <i>O</i> -methyl glucuronic acid
25. MWCO	Molecular weight cut off
26. nC	Nano chromatogram
27. NERL	National Energy research laboratory
28. ¹³ C- NMR	Carbon nuclear magnetic resonance
29. OD (o.d)	Oven dry
30. PEG	Polyethylene-glycol
31. <i>p</i> NPA	<i>para</i> -nitrophenol α -L-arabinofuranoside
32. RSM	Response surface methodology
33. SDS/PAGE	Sodium dodecyl sulphate -polyacrylamide gel electrophoresis
34. TAPPI	Technical analysis for the pulp and paper industry
35. TMS	Tetramethylsilane
36. V _{max}	Maximum velocity
37. δ	Chemical shift
38. ¹³ C-CP/MAS NMR	Carbon resonance Cross-Polarisation/Magic Angle Spinning nuclear magnetic resonance
39. 2xMM	Double minimum media

Chapter 1: Introduction and literature review

1.0 INTRODUCTION

Biodegradability coupled with biocompatibility make polysaccharide preferred polymers for many industrial applications compared to synthetic polymers. Polysaccharides, which are made of 1,4-glycosidic linked sugar units in their main backbone chain such as starch, cellulose and xylans, have a natural ability to form insoluble hydrogels and biofilms. Such hydrogels and biofilms can be predesigned during formation to suit various applications including additives, implantation matrices and novel carrier and delivery systems in the pulp and paper, packaging, pharmaceutical and cosmetic industries. However, production of the insoluble hydrogels and biofilms is relatively easier with starch and cellulose than with xylans. This is because starch and cellulose are homopolymers of higher molecular mass such that hydrogen bonding between and within the polymers occurs so easily, leading to the precipitation or gelation state. In contrast, xylan is a heteropolymer with higher degree of substitution and is of lower molecular mass than cellulose and starch, therefore, does not precipitate readily. Consequently, these characteristics limit the industrial application of the xylans.

The need to utilise insoluble xylan has increased because cellulose and starch have many other competing uses in the food, pharmaceutical, paper and textiles industries. For instance, the cellulose is used as source of fibre for the pulp and paper industry furthermore, both cellulose and starch are used as additives in the food, pharmaceutical, pulp and paper and textiles and sources of fermentable sugar for biofuels production. Yet, xylan is available in abundance, accounting for 25-35% biomass dry weight in higher plants (Timell, 1967). In addition, xylans have added advantages over other natural polymers because they possess antiviral and antitumor activities relevant for pharmaceutical and biomedical applications. However, a large proportion of the available xylan is currently wasted in lignocelluloses process waste streams with little prospects for recovery and addition of value.

The formation of insoluble xylan hydrogels and biofilms can be achieved by selective removal of side sugar residues that are attached to the main xylan chain. Xylans are

often substituted with either by α -L-arabinofuranoside (arabinose) or 4-O-methylglucuronic acid (MeGlcA)/glucuronic acid (GlcA) or both side chains. Although physical and chemical methods are available for the removal of the side chains, such methods are inefficient because they depolymerise and degrade the main xylan chain. Consequently, the ability of the xylan polymers to become insoluble and function to the same level as starch and cellulose polymers is compromised. Furthermore, the degraded xylan fractions produce organic and chromophogenic compounds, which upon their disposal, contribute to environmental pollution. Therefore, in order to prepare the xylans into insoluble hydrogels and biofilms from xylans, there is need to develop an effective technology for selective removal of the arabinose and MeGlcA side chains. The targeted removal of the side chains would permit customised precipitation of the xylans according to end-use requirements.

Targeted removal of the arabinose and MeGlcA side chains can be achieved by use of speciality enzymes, which selectively remove the side chains under controlled conditions according to their substrate specificity. The use of such enzymes is more advantageous for precipitation of xylans compared to the use of chemical and physical methods because the main xylan chain is not degraded. Moreover, enzymes that degrade xylans occur in nature, as such, have reduced impact on the environment. However, most of the available enzymes for removing arabinose and MeGlcA side chains are more effective when they work in a consortium with xylan main chain degrading enzymes than alone and as such, do not have the ability to precipitate the xylans. Enzymes capable of removing the arabinose and MeGlcA side chains from polymeric xylans in the absence of the main chain degrading enzymes would provide an effective and flexible technology for reducing the solubility of the xylans leading to formation of insoluble hydrogels and biofilms. So far, α -L-arabinofuranosidase (EC3.2.1.55) (AbfB) from *Aspergillus niger* described by Tagawa and Kaji (1988) and Gielkens et al. (1997) and the α -D-glucuronidase AguA (EC3.2.1.139/131) from *Schizophyllum commune* described by Tenkanen and Siika-aho (2000) and α -D-glucuronidase from *Pichia stipitis* described by Ryabova et al. (2009) are unique enzymes isolated from fungal sources that have demonstrated ability to selectively remove arabinose and MeGlcA side chains respectively, from polymeric xylan causing it to precipitate. However, in nature AbfB and AguA are produced in consortium with xylanases, which compromises their use for precipitation of xylans.

Consequently, direct application of the AbfB and AguA in precipitation of water soluble polymeric xylans cannot be done without purification. This study focused on developing enzyme technology based on recombinant α -L- arabinofuranosidase and purified α -D-glucuronidase with polymeric xylan substrate specificity for reducing solubility of the water soluble xylans. The process involved development of the recombinant fungal expression system for production of the AbfB that was free of xylanase activity (Chapter 4). Therefore, recombinant AbfB and purified AguA were assessed for selective removal of arabinose and MeGlcA side groups from polymeric xylan substrates extracted from feedstocks grown in Southern Africa and xylans obtained from commercial sources (Chapter 3, 5 and 6). In addition, the industrial applications of the recombinant AbfB and purified AguA in transforming xylans into nanohydrogels for use as speciality coating for cellulosic surfaces and as encapsulation matrices for slow delivery of bioactive agents were studied (Chapter 7 and 8).

1.1 LITERATURE REVIEW

1.1.1 Xylan sources and supply

Xylan is one of the major hemicelluloses in higher plants (Timell, 1967). Other hemicelluloses found in plants include mannans (galactoglucomannans and glucomannans), which are commonly found in softwoods (Timell, 1967). Furthermore, hemicelluloses are present in plants as mixed linked β -glucans, xyloglucans and arabinogalactans (Ebringerová, et al., 2005). The xylan can be extracted from hardwood (angiosperms), softwoods (gymnosperms) and non-wood sources such as grasses and cereals, belonging to the *Gramminae* family (Ebringerová and Heinze, 2000). The hemicellulosic composition of the hardwood sources is about 20-30% of biomass (on dry weight basis) (Sjöström, 1993). In hardwoods (*Eucalyptus grandis*) and some fibrous grasses (bamboo), the xylan may constitute up to 90% of the available hemicelluloses (Ebringerová and Heinze, 2000; Ebringerová, 2006). In softwood and grasses, the hemicelluloses content of the cell walls lies between 8 and 40% of the biomass (on dry weight basis), 50% of which, could be xylan (Kuhad et al, 1997; Ebringerová, 2006). The cellulose and lignin contents in most plant sources range between 40 and 50% and 20-30% of biomass dry weight respectively (Timell, 1967; Kuhad et al., 1997). However, in other annual

plants, the lignin content could be as low as 11% (Timell, 1967; Wilkie, 1979; Kuhad et al., 1997). In addition to cellulose, lignin and hemicelluloses, the lignocellulosic materials contain proteins, pectins, inorganic components (ash) and extraneous substances, which collectively are grouped as extractives (Browning, 1970). The extractives and lignin interfere with extractability and modification of the xylans (Ebringerová et al., 2005).

Sources of xylans include: *Eucalyptus grandis*, *Pinus patula* and grasses such as *Saccharum officinarum* (sugarcane) process residue (bagasse) and *Bambusa balcooa* (giant bamboo). The bamboo and *E. grandis* contain long cellulosic fibers (1.5–3.2 mm), as such, are preferred feedstock for pulp and paper making in most developing countries including Brazil, India and China (Scurlock et al., 2000; Whish-Wilson and Maley, 2002; Gielis, 2002). In South Africa, the *E. grandis* and *P. patula* are currently the major raw materials for the pulp and paper and timber industries. Unlike in other pulp and paper producing countries, the *E. grandis* and *P. patula* in South Africa are produced on a relatively short rotational basis, which would provide sustainable supply of feedstocks for xylan extraction.

The yield of timber in South Africa is approximately 16.86 million metric tons (MT) per annum of which 8.69 million MT is softwoods, mainly *P. patula* and 8.17 million MT constitute hardwoods, predominantly *E. grandis* (DME, 2004). The South African pulp and paper industry utilises about 10 million MT of the harvested timber (DME, 2004). Furthermore, additional sources of xylans include: 6.05 million MT per annum biomass waste, which is generated from forestry and timber process residues and additional 11.47 million MT per annum of biomass residue from other industries of which, 6.136 million MT is bagasse (DME, 2004). About 90% of the bagasse is used for generation of energy at the South African sugar mills but with use of energy efficient mills, 45% sugarcane bagasse could be spared for other uses (Paterson-Jones, 1989), which can include xylan extraction. Therefore, a combination of wood, forestry products including bamboo plantations (estimated to be over 6 000 ha) (RSA-LT, 2003, The Biomass Corp., 2009) and the bagasse, provides South Africa with capacity to produce approximately 4.7 million MT of xylan per annum. The xylan could be co-produced with the conventional pulp and paper and timber products and can therefore provide a platform for establishing an integrated lignocellulose based biorefinery. The

current status quo suggests that xylan quantities of approximately 4.7 million MT are either incinerated during recovery of pulping chemicals or are dumped in land fill sites. The energy generated from incineration of xylan is of lower economic value compared to biomaterials that can be prepared from the xylans. The heating value of xylan is approximately 13.6 MJ/kg, which is 50% the energy value from lignin (Tunc et al., 2008^a).

1.1.2 Structure and chemical composition of cell wall components

Cellulose and xylans are polysaccharides present in close association in the plant cell wall matrix that is encrusted by lignin (Fengel and Wegener, 1989; Sjöström, 1993). The cellulose main backbone consists of regular linear homopolymer of (1→4)- β -glycosidic linked D-glucopyranose monomer units, which are arranged into microfibrils with diameters ranging from 10-25 nm (Timell, 1967; Fengel and Wegener, 1989). The cellulose has a degree of polymerisation (DP) in the range between 5 000 and 10 000 sugar units (Timell, 1967; Fengel and Wegener, 1989). The high molecular mass and lack of branching enhances gelation of cellulosic (Kajiware and Miyamoto, 2005).

Lignin is present in the plant cell wall as naturally occurring binding agents (Sjöström, 1993). The lignin component is amorphous substance that is partly aromatic and contains methoxy groups, aliphatic and phenolic hydroxyls (Browning, 1970). The principle structural elements of lignin present in plant cell walls include *p*-coumaryl alcohol (*p*-hydroxyphenyl propanol), coniferyl alcohol (guaiacyl propanol) and synapyl alcohol (syringyl propanol) (Jeffries, 1994). The lignin in hardwoods is present as a copolymer of guaiacyl and syringyl units in 4:1 ratio, whereas, in softwood the principal lignin content is a polymerization product of coniferyl alcohol (guaiacyl lignin) (Timell, 1967; Sjöström, 1993; Jeffries, 1994). The lignin in lignocellulosic materials of the Gramineae family is present in all the three forms (Wilkie, 1979).

The xylans are comprised of (1→4)- β -linked-D-xylopyranosyl backbone (Fengel and Wegener, 1989). However, xylans display polydispersity and polymolecularity to much greater extent than cellulose (Joseleau et al., 1992). Extensive reviews of structural and chemical composition of xylan residues from wood have been presented

by Timell (1967), Browning (1970), McNeil, et al. (1984), Fengel and Wegener (1989), Joseleau (1992) and Sjöström (1993), whereas, those from grasses, have been presented by Wilkie (1979). It is established that the physical and chemical composition of the xylans depend on the botanical source, morphological part, physiological age and growing conditions (Timell, 1967; Joseleau et al, 1992) and the isolation methods (Browning, 1970). Some of the xylan structures occurring in nature have been presented in Figure 1.1. Notably, hardwood xylans (Fig. 1.1a) has about 70% of the xylose units acetylated at C2 and C3 and may carry single terminal GlcA or MeGlcA substituents that are α -(1 \rightarrow 2) glycosidic linked at the xylose units in a 10:1 (xylan:MeGlcA/GlcA) ratio (Timell, 1967; Fengel and Wegener, 1989). Therefore, the hardwood xylans are referred to as *O*-acetyl-4-*O*-methylglucuronoxylans (Ebringerová and Heinze, 2000). The hardwood xylans may also carry arabinose as non reducing end groups, which constitute 2% of the xylan (Fengel and Wegener, 1989).

The xylans present in softwoods and some grasses including sugarcane bagasse and bamboo (Fig. 1.1b) are referred to as arabinoglucuronoxylans because they carry both MeGlcA and arabinose side chains, which are linked by α -D-(1 \rightarrow 3)-glycosidic bond to the xylan backbone (Wilkie, 1979; Fengel and Wegener, 1989; Ebringerová and Heinze, 2000). The softwood xylans are not acetylated but have higher degree of substitution (3-6:1) of MeGlcA to xylose units than hardwoods (Ebringerová and Heinze, 2000). In addition, the xylans from grass sources may carry xylose side chains described as *O*-D-xylopyranosyl-(1 \rightarrow 2)-*O*-L-arabinofuranosyl-(1 \rightarrow 2)-L-arabinose (Wilkie, 1979, Yoshida et al., 1998). In cereal grains, such as wheat and oat spelt (Fig.1.1c), the xylans contain mainly arabinose substituents, which are either single or doubly substituted and are called arabinoxylans (Wilkie, 1979). The hardwood, softwood and grass xylans may also carry additional short side chains consisting of arabinose, galactose, rhamnose and xylose. In some plants, the xylans are esterified with ferulic or *p*-coumaric acid (Ebringerová and Heinze, 2000).

1.1.3 Xylan interaction with other cell wall components

The interaction of xylan with lignin and cellulose, both inside and outside the plant cell wall matrix is both through molecular bonding and physical association (Ebringerová and Heinze, 2000). The xylan-cellulose-lignin interactions depend

mostly on the degree of xylan polymerisation and substitution and substitution pattern (Sjöström, 1993; Henriksson and Gatenholm, 2002^b; Westbye et al., 2006; Danielsson, 2007; Köhnke and Gatenholm, 2007; Dammström et al., 2009). Less substituted xylan is often tightly bound to cellulose whereas; highly substituted xylan is tightly bonded to lignin through ester and ether linkages (Joseleau et al., 1992). Ester linkages are formed between xylan and lignin via MeGlcA (Talahashi and Koshijima, 1988) whereas; the ether linkages are formed through arabinose side groups (Timell, 1967; Wilkie, 1979). Consequently, such lignin-xylan crosslinkages result into formation of lignin-xylan-cellulose composites termed as lignin-carbohydrate complexes (LCCs) (Joseleau et al., 1992; Argyropoulos and Menachem, 1997; Ebringerová and Heinze, 2000; Lawoko et al., 2006^{ab}; Patel et al., 2007). Such highly substituted xyans crosslinked to the lignin residues are able to adsorb onto cellulosic material (Henriksson and Gatenholm, 2001), which increases the water holding capacity (Linder et al., 2003^a) and the tendency of the cellulosic material to discolour with aging (Lee et al., 1989). The methods for breaking the LCCs are not well developed (Ebringerová et al., 2005). For instance, the LCCs were not completely hydrolysed neither by alkaline nor both acid hydrolysis methods and through borohydride reduction (Morrison, 1974; Yang et al., 2005). The lignin complex formed with corn cob xylan did not change during a 12 h treatment in sulphuric acid (1.0 g/l H₂SO₄) at 60°C Yang et al., 2005). Other workers have used xylan main chain-attacking enzymes such as xylanases, to break down the LCCs (Bajpai, 2004). Although positive results have been obtained with such enzyme treatments, the use of oxidising agents has remained the common method for the removal of the LCCs (Timell, 1967; Browning, 1970; Jeffries, 1994). However, the removal of the LCCs by oxidation could result into unavoidable depolymerisation and damage of the structure of the xylan. As a result, the xylan bound in LCCs may not be available for customised modification of its functional properties.

The intimate interaction between xylan and cellulose in LCC is of concern, in particular, during the delignification and bleaching stages of the kraft pulp and paper making processes (Henriksson and Gatenholm, 2001). This is because the xylan substituted with uronic acid introduces chromophores in the form of 4-deoxy-β-L-threo-hex-4-eno-pyranosyluronic acid hexenuronic acid (hexenuronic acid (HexA)) groups that cause yellowing of cellulosic pulp and paper products with aging (Jiang et

al., 2006). The HexA adsorbed onto pulp fibres increase consumption of bleaching chemicals beyond amounts required for removal of residual lignin by up to 3-7 Kappa units (Buchert et al., 1995; Li et al., 1997; Vuorinen et al., 1999; Shatalov et al., 2004; Siika-aho et al., 2004; Danielsson et al., 2006). Therefore, pre-extracting xylan, in particular xylan substituted with uronic acids side groups prior to pulping the feedstocks would enhance the bleaching process and reduce the amount of chemicals and time required to achieve desirable results (Ragauskas et al., 2006).

However, xylan-lignin-cellulose interactions can be used positively under controlled conditions as a basis for production of novel lignin-xylan-cellulose composite materials with new properties and functionalities (Ebringerová et al., 2005). It has been shown that the interaction between xylan and lignin facilitates adsorption of water soluble xylan onto cellulosic materials (Linder et al., 2003^{ab}), leading to formation of xylan-lignin-cellulose composites that are hydrophilic. The tendency for the xylan to permanently adsorb onto cellulosic surfaces presented a basis for use of the xylan as natural coating material that modified the surfaces properties (Wilkie, 1979; Henriksson and Gatenholm, 2001; Håkansson et al., 2005). The xylan with surface charges, regulated the cellulose fibrillogenesis both when inside and outside the plant cell wall matrix, which prevented aggregation of the cellulose microfibrils (Dammström et al., 2009). In addition, the xylan adsorbed onto cellulose fibres protected the cellulosic fibres from acid hydrolysis both at fibre and fibril levels (Håkansson et al., 2005; Kayserilioğlu et al., 2003). The adsorbed xylan on the cellulosic surface imbibed water contributed to fibre swelling causing internal lubrication, loosening of the fibre structure and improved fibre to fibre bonding of the cellulose microfibrils (Walker, 1965; Meller, 1965; Whitney et al., 1995; Dahlman, et al., 2003; Kabel et al., 2007).

Many workers have demonstrated the xylan-cellulose interaction phenomena on various cellulosic materials including never dried and dried pulp fibres (Walker, 1965; Köhnke and Gatenholm, 2007; Köhnke et al., 2008), bleached and unbleached pulp from softwood kraft pulp (Ramírez et al., 2008), chemi-thermomechanical pulps (CTMP) (Henriksson and Gatenholm, 2002^a), plant cotton fibres (Henriksson and Gatenholm, 2001), cotton linters (Walker, 1965), cotton whiskers (Saxena and Ragauskas, 2009; Saxena et al., 2009), recycled fibres (Arndt and Zelm, 2008) and

bacterial cellulose (Kabel et al., 2007). In addition, the interaction of the xylan and the lignin were used for production of compounds of higher molecular mass for use in paper surface sizing, briquette binding and either as corrugated carton or board adhesives (Van Der Klashorst, 1989).

The xylan that adsorbed on the cellulosic surfaces have been in the form of nano and microhydrogels, which inherently can swell to different degrees under varying solution conditions (Henriksson and Gatenholm, 2001; Kishida and Ikada, 2002; Linder et al., 2003^a; Numan and Bhosle, 2006; Kabel et al., 2007). Such hydrogels are useful for production of biomaterials such as those used for production of tissue replacements, immunoisolation membranes, biosensors, functional packaging, sanitary and textile products (Cole et al., 1992; Kishida and Ikada, 2002; Numan and Bhosle, 2006; Silva et al., 2007). In addition, the xylan hydrogels have specific application as encapsulation or entrapment matrices for immobilisation and targeted sustained release of bioactive substances or other compounds in nano and micro sized dosages. For instance, xylan hydrogels produced through esterification using carboxylic acid with N, N'-carbonyldiimidazole (CDI) that was sulphated using SO₃ and DMF, were used for encapsulation and slow release delivery of ibuprofen (Daus and Heinze, 2009). In another application, xylan hydrogel coatings enabled development of novel packaging material with restricted gas permeability known as xylophane (Gröndahl et al., 2004; Gatenholm, et al., 2008) and xylan-cellulosic composites with reduced specific water transmission of up 362% (Saxena and Ragauskas, 2009). Therefore, the xylan-cellulose interaction coupled with the formation of the xylan hydrogels provides the basis for production of speciality coating and encapsulation matrices to produce diversified functional cellulosic products and slow delivery systems.

1.1.4 Isolation of polymeric xylans

Therefore, successful development of the enzyme technology for reducing xylan solubility would depend on isolation of water soluble polymeric xylans from the lignocellulosic materials that is close to its native form (i.e. xylan fractions with degree of polymerisation (DP) of at least >10 xylose units and have side groups attached). The xylan extracted closer to its native form provides the opportunity for custom modification of its functional properties (Ebringerová and Heinze, 2000).

Xylans extracted for enzymatic modification are required to have the necessary binding sites for the removal of the side chains to occur. The extractability of the xylans from hardwoods, softwoods and grasses, is among other factors, influenced by content and type of lignin, and side group and degree of branching of the xylan itself (Timell, 1967; Ebringerová et al., 2005). The extractability of xylans from hardwoods is considered relatively high compared to that of arabinoglucuronoxylans from softwoods and some grasses and is attributed to mainly less degree of branching, lignin type (guaicyl and syringyl or mixture) and relatively lower lignin levels (Timell, 1967; Jeffries, 1994). Similarly, the extraction of arabinoxylans from annual plants is relatively easier than the extraction of glucuronoxylans and arabinoglucuronoxylans from woods (Timell, 1967) because of being monosubstituted with mainly arabinose side chains that are less resistant to alkali cleavage than MeGlcA (Sjöström, 1993). The excessive branching of the arabinoglucuronoxylans that forms crosslinkages with lignin through arabinose and MeGlcA side groups makes it difficult to extract high yields of pure xylan fractions (Timell, 1967).

Xylans can be isolated directly from plants but could also be recovered as a by-product from lignocelluloses process waste streams such as those from kraft pulping and dissolved pulp production waste streams. Isolation of xylans during kraft pulping, delignification and solubilisation of the xylans occur simultaneously at high alkaline (16-20% NaOH charge [pH 13-14]) and high temperatures (155-175°C) conditions (Sjöström, 1993). Such kraft pulping conditions cause degradation of the cellulose and the xylans as a result of end-wise peeling reactions (Sjöström, 1993), which during isolation of glucuronoxylans produces 4-deoxy- β -L-threo-hex-4-eno-pyranosyluronic acid, hexenuronic acid (Teleman et al., 1995). It has been shown that 87% of uronic acids present in xylan were converted to hexenuronic acid during kraft pulping of *Eucalyptus globules* at temperatures higher than 110°C (Simão et al., 2005). Other workers have shown that pulping of non wood fibrous materials using 25% NaOH and 40% ethanol (EtOH) at 130-150°C resulted into loss of 90% of uronic acid moieties from the xylan, which corresponded to formation of about 84% of hexenuronic acid (Shatalov and Pereira, 2004). Evidently, the use of strong alkali charge of $\geq 16\%$ and high temperatures of $>160^\circ\text{C}$ for delignification during kraft pulping does not favour extraction of polymeric xylans or preservation of xylans in a format suitable for biopolymer applications.

The xylan recovered from kraft waste streams may not conform to the structural and purity requirements for enzymatic precipitation due to degradation and contamination from pulping chemicals, and lignin and cellulosic degraded products (Sjöström, 1993; Lisboa et al., 2005). Although it might be possible to recover some of the xylans as pure polymeric fractions from the kraft pulp waste streams (i.e. xylan fractions obtained by membrane separation) (Glasser et al., 2000; Ebrígrenová and Heinze, 2000; Jönsson et al., 2008), such source of xylan would not be economical because of the relatively high recovery costs. For instance, Vu et al. (2003) showed that only 4-7% of polymeric xylan could be recovered from the black liquor obtained from kraft pulping of bamboo (*Bambusa procera acher*). Consequently, xylan fractions with low and widely distributed molecular mass are produced, which may not suit enzymatic precipitation for production of homogenous xylan hydrogels and biofilms. Instead, the degraded xylan fractions would degrade further, thus releasing organic acids that result in increased chemical oxygen demand (COD) in the lignocelluloses waste streams.

Xylans with physical and chemical structures suitable for enzymatic modification can be pre-extracted from feedstocks prior to processing of the feedstocks such as in kraft pulping (Ragauskas et al., 2006; Al-Dajani and Tschirner, 2008). The potential to selectively isolate xylans from the cellulose–lignin plant matrix could be based on differences in their molecular mass or degree of polymerisation (DP), degree of branching and their dissolution and precipitation behaviours in certain solvents (Browning, 1970; Horvath, 2006). Therefore, xylan because of its DP, which is lower (ranging from 60-200 sugar units) has characteristic dissolution in alkali solution and is completely hydrolysable in dilute acids compared to the cellulose with DP in the range of 5000-10000 (Browning, 1967; Sjöström, 1993). On the other hand, the differences in the dissolution and precipitation properties of xylan and lignin fractions are not always apparent such that some fractions of lignin and xylan dissolved and precipitated together in alkali solutions (Schooneveld-Bergmans et al., 1999). Consequently, it is difficult to isolate xylan fractions completely free of lignin fractions.

A number of selective isolation technologies for xylans have been developed based on the differences in the dissolution properties of the xylan in acid, water, alcohol and alkali solvents. Such methods have included mild alkali/acid extraction (De Lopez et al., 1996, Glasser et al., 2000; Gabrielii et al., 2000; Sun et al., 2004^{ab}; Höije et al., 2005), solvent extractions (organosolv pulping) (Pan et al., 2005, Höije et al., 2005; Xu et al., 2006) hydrothermal aquasolv extraction (Garrote et al., 2004; Bobleter, 2005, Mosier et al., 2005; Vázquez et al. 2005; Benkő et al., 2007; Roos et al., 2009), and ionic solvents (ionic liquids) extractions (Roger and Seddon, 2003; Lee et al., 2009). However, the yields, purity, degree of polymerisation and substitution and substitution pattern of the extracted xylans have differed from method to method and between the feedstocks. Therefore, the choice of appropriate isolation method would depend on the requirements for the subsequent methods for modifying the xylan and the intended end use of both the xylan products and the cellulolignin residues.

(a) Hydrothermal aquasolv xylan extraction

Hydrothermal aquasolv xylan extraction, which in this context includes methods such as supercritical water or steam-aqueous treatments (Solvolysis) and microwave irradiation (Kitchaiya et al., 2003; Garrote et al., 2004; Mosier et al., 2005; Benkő et al., 2007; Tunc and Heiningen, 2008^{ab}), separates the xylan from the cellulose and lignin matrix based on auto hydrolysis effect. The hydrothermal method involves exposure of lignocellulosic materials to high temperatures (130-250°C), which are sometimes combined with exposure to high pressures (up to 25 bar) for short periods (Sjöström, 1993; Palm and Zacchi, 2003; Mosier et al., 2005, Tunc and Heiningen, 2008^{ab}). During the hydrothermal treatment, hydronium-catalysed breakdown of the glycosidic and cleavage of ester bonds of the polysaccharides occur which released acetyl groups and organic acids (Garrote et al., 2004; Vázquez et al. 2005; Griehl et al., 2006; Benkő et al., 2007, Roos et al. 2009). Unfortunately, the hydrothermal extraction procedures did not favour extraction of high yields of polymeric xylan. For example, Tunc and Heiningen, (2008^{ab}) reported yield of xylan of approximately 9% (oven dry mass basis) from hydrothermal treatment of mixed southern hardwoods. Whereas, Roos et al. (2009) reported 9% yields of xylan in oligomeric form after hydrothermal treatment of barley husks. Therefore, the hydrothermal xylan extraction process from both technical and economic point of views may not be a suitable method for extracting xylan for enzymatic modification.

(b) Ionic solvent xylan extraction

Ionic liquids are liquids that entirely consist of cations and anions (Pu et al., 2007) and have a melting point at or below 100 °C (Lee et al., 2009). In addition, the ionic liquids have low volatility and high thermostability, as such; they are considered green solvents (Pu et al., 2007). The ionic solvents extraction methods have been studied for fractionation of lignocellulosic material instead of using conventional solvents such as alkali/ acid and ethanol. Such studies have particularly focused on selective solubilisation of lignin (Rogers and Seddon, 2003; Pu et al., 2007; Lee et al., 2009; Fu et al., 2010) rather than isolation of xylan. However, there is potential to use the ionic liquids for selective isolation of xylan because of the possibility to custom design the ionic liquids that are tailor made for a specific lignocellulosic product profile (Park and Kazlauskas, 2003; Lee et al., 2009). Recovery of polymeric xylan (xylan type not specified) liquefied in ionic liquids of up to 80% with ethanol as an antisolvent was possible (Nakamura et al., 2010). However, the efficiency of the ionic solvent for extraction of the xylan would also depend on the recyclability of the ionic liquids, which are supplied at high cost.

(c) Mild acid xylan isolation

Mild acid based xylan isolation methods are preferred to strong acid treatment in order to increase yield and purity of the xylan fractions extracted in oligomeric form (Browning, 1967; Ebrigenová and Heinze, 2000; Yang et al., 2006). Therefore, the mild acid methods do not suit extraction of xylan in polymeric form. For example, at acid concentration of 0.05-1% (by wt), the xylan extracted was predominantly in oligomeric form (e.g. degree of polymerisation (DP) of between 1 and 4) (Liu and Wyman, 2004; Yang et al., 2006). In another study, the use of dilute sulphuric acid (1.08% w/w) at 185°C for 4 min in a steam explosion reactor yielded 76% of the xylan as monomeric xylose, 9% oligomeric xylose and 5% furfural (Kim et al., 2001). Xylan yield, mostly in oligomeric and monomeric form of up to 79% (w/w) of the original xylan content was reported by Yang et al. (2006) from dilute acid pre-treatment of aspen that was followed by dry-steaming. Noteworthy, the xylan fractions extracted under acid conditions have most of the terminal side groups

removed (Wilkie, 1979) and are contaminated with acid soluble lignin and furfural, which might be toxic to xylan hydrolysing enzymes (Yang et al., 2006).

(d) Mild alkali xylan isolation

Higher yields of polymeric xylans from lignocellulosic materials are extracted with mild alkaline-low temperature extraction methods (<16% alkali charge, temperature <100°C) compared to the mild acid and hydrothermal methods. The contributing factors to such high xylan yield include reduced xylan degradation (Ebrigenová and Heinze, 2000). For instance, xylan, predominantly in polymeric form was extracted with yields of 83% with the use of mild alkali-low temperature extraction methods on barley husks (Höije et al., 2005) and of 57% on barley straw (De Lopez et al., 1996). Due to the differences in dissolution of xylan and cellulose in alkali solutions, the mild alkali xylan extraction methods could be manipulated to allow large amounts of xylans to be selectively extracted from the lignocellulosic material in different fraction sizes (Browning, 1967). Under mild alkaline condition, the integrity of the cellulosic residue is preserved, which provides potential for co-production of the xylan with pulp and other value added products in a biorefinery context (De Lopez et al., 1996; Ragauskas et al., 2006; Al-Dahani and Tschirner, 2008). The pre-extraction of the xylan prior to pulping, reduced the duration kraft cooking by 10 min and the sulphur charge (sodium sulphide (NaSH)) by 5.5-8.0 kg per MT of aspen chips respectively (Al-Dahani and Tschirner, 2008). In addition, the mild alkali xylan extraction performed prior to pulping would provide feedstock residues with new functional properties. The mild alkali-low temperature extraction methods involve three major steps, which are delignification, solubilisation and recovery (Browning, 1967; Ebrigenová and Heinze, 2000; Gabrielii et al., 2000; Sun et al., 2004). The mild alkaline extraction methods are often performed in combination with pre-isolation and post-isolation treatments that involve addition of additives and fractionation procedures.

(i) Pre-treatment of lignocelluloses for xylan extraction

The pre treatment is done prior to mild alkali extraction of xylan to remove extractives have included: benzene, methanol/alcohol, ammonium oxalate, oxalic acid/ editic acid (ethyldinitrilo) tetraacetic acid [EDTA]) and pectins (Wilkie, 1979;

Vázquez, 2000; Höije et al., 2005). In other pre-treatment procedures, enzymatic methods were used to remove starch and proteins (Sun et al., 2004^b).

(ii) Delignification

Extraction of xylans from softwoods and heavily lignified grasses often requires a delignification step unlike hardwoods (Timell, 1967; Wilkie, 1979). Therefore, the xylan can be extracted directly from hardwood without delignification (Timell, 1967; Wilkie, 1979). During delignification, the lignocellulosic materials are subjected to oxidative agents that unavoidably cause degradation and partial depolymerisation of the cell wall polysaccharides (Timell, 1967; Wilkie, 1979). In some xylan extraction processes, the delignification was achieved through reaction of the feedstock with chlorine gas, digesting with acetic acidified sodium chlorites and sodium sulphite solutions (Wilkie, 1979; Coimbra et al., 1995).

Among the delignification methods, the chlorite method has been the most common and effective (Browning, 1970). The chlorite delignification method was used during extraction of xylan from barley husks, which resulted in reduction of the lignin content from 11.5% to 2.5% as opposed to organosolv delignification method whereby lignin content was reduced to 3.9% (Höije et al., 2005). However, the chlorite delignification method raises environmental concerns because of the release of chlorine to the environment (Ebringerová et al. 2005). Therefore, there is growing interest in developing chlorine free delignification technique (Höije et al., 2005) that would have less impact to the environment, and the functioning of the enzymes.

(iii) Xylan solubilisation

The xylan solubilisation under alkaline conditions is often achieved with either sodium hydroxide (NaOH) or potassium hydroxide (KOH) (Timell, 1967). However, the KOH is often preferred over NaOH because it has shown to give maximum xylan yield with minimum contamination from glucomannan (Timell, 1967). Hardwood xylan could be isolated by use of the KOH xylan extraction method to a maximum yield of up to 80% (Timell, 1967). Furthermore, the potassium acetate formed upon reacting the KOH with acetic acid during neutralisation of the xylan extract is more soluble in the alcohol used for precipitating the xylan extract than the corresponding

sodium acetate formed with NaOH (Sjöström, 1993). Although the KOH based xylan extraction appear attractive for extraction of polymeric xylans, the comparative cost of the KOH to NaOH makes the latter to be the economically preferred option. The mild alkali-low temperature methods have been found suitable for co-production of xylans with other lignocellulosic based products. De Lopez et al. (1996) found optimal conditions for extraction of the xylan (57%) for integration with soda-anthraquinone pulping of barley straw were 13% NaOH charge extracted at 60°C for 2 h. Al-Dajani and Tschirner (2008) reported xylan yield of 40-50 kg per MT of wood chips extracted with NaOH at a charge between 1.67 and 2.08 M and temperature between 50 and 90°C for 4 h that was co-produced with pulp in yields similar to traditional pulping.

During solubilisation of xylans, additives such as sodium borohydride were added to reduce dissolution of six carbon sugars during alkaline xylan extraction, which improved the purity of the extracted xylans and preservation of the cellulosic residue (Browning, 1967; Höije et al., 2005). Furthermore, alkaline solutions containing hydrogen peroxide (H_2O_2) were used to facilitate delignification, solubilisation and to improve whiteness of the extracted xylan from wheat straw (Sun et al., 2000). In addition the use of sequential mild alkali performed in combination with ultrasonic treatment yielded xylan of up to 90% (Sun et al., 2004b). Furthermore, the use of the alkaline solutions of hydrogen peroxide resulted in xylan yield of up to 91% from wheat straw, which was accompanied by 86% release of lignin. Similarly, in a study by Brienzo et al. (2009) the use of 6% H_2O_2 gave xylan yield of 86% from sugarcane bagasse, which had lignin content of 5.9% and was light coloured.

Alternatively dissolution of polymeric xylans from lignocellulosic materials is possible with neutral solvents such as dimethyl sulfoxide (DMSO). The DMSO has been effective for isolation of polymeric xylans from wood holocellulose with minimal deacetylation (Timell, 1967; Sjöström, 1993). However, the xylan yield hardly exceeds 50% of the available xylan (Timell, 1967). The use DMSO failed to break linkages between lignin and xylan in grasses (Wilkie, 1979), which resulted in lower yields. The lower yields from wood sources and the failure to produce lignin-free xylan fractions, renders the DMSO method not economically suitable for large scale xylan extraction for enzyme modification.

(iv) fractionation of xylan

The xylan fractionation procedures for recovery of isolated xylan fractions have involved precipitation, ultrafiltration, centrifuge, diafiltration or a combination of these (Persson et al., 2005; Höije et al., 2005; De Lopez et al. 1996). Other methods have used activated carbon, ion exchange, reverse osmosis and membrane separations (Glasser et al., 2000; Ebringerová and Heinze, 2000; Jönsson et al., 2008). Highly substituted and complex xyans were found to be poorly fractionated by the ethanol precipitation methods (Wilkie, 1979; Sun et al., 2004^b). Therefore, it is possible to obtain xylan with features that would suit enzymatic modification. The pure xylan fractions from such purification procedures are provided by a narrow range of molecular weights and constant ratio between two sugar residues. Fractionation by precipitation of the xylan solubilised in alkaline solution is achieved by addition of cations or solvents such as acetone or ethanol upon neutralisation or acidification (Wilkie, 1979). The degree of polymerisation profile of the fractionated xylan residues from the ethanol precipitation procedure depends on the ethanol concentration (Schooneveld-Bergmans et al., 1999). The graded fractionation procedures to yield homogeneous xylan fractions were achieved by controlling operating factors such as solvent concentration and temperature (Coimbra et al., 1995; Schooneveld-Bergmans et al., 1999; Xu et al., 2006; Peng et al., 2009).

1.1.5 Modification of solubility properties of xylan

The solubility of the xylan is determined by the degree of polymerisation, substitution and the substitution pattern (Ebringerová and Heinze, 2000; Hornig and Heinze, 2008). Therefore, targeted alteration of such xylan features would modify the solubility properties. It has been reported that the degree of xylan substitution does not necessarily precipitate the xylan unless it is accompanied by attraction forces within and between the xylan polymers (Linder et al., 2003^a; Gröndahl et al., 2004; Westbye et al., 2006; Silva et al., 2007; Kabel et al., 2007; Ramírez et al., 2008; Köhnke et al., 2009). Therefore, the key factor for the precipitation of water soluble xylan was attributed mainly to the altered distribution pattern of the side groups along the xylan main chain rather than to the reduced degree of substitution alone (Linder et

al., 2003^{ab}; Kabel et al., 2007). Therefore, the selective removal of the side chains in order to precipitate the xylans, is a major challenge in processing of lignocelluloses (Ebringerová and Heinze, 2000; Lindblad et al., 2001). Consequently, the use of xylans in insoluble form as biodegradable coating material, entrapment matrix and slow delivery systems is limited (Ebringerová and Heinze, 2000).

Although the precipitation and self assembling behaviour of annual plants (arabinoxylans) was exhibited with arabinose to xylose ratio of 0.30 (Höije et al., 2008), in hardwood xylan (glucuronoxylan) such effect was observed with relatively highly substituted xylan but possessing at least a minimum of 15 unsubstituted consecutive xylose units (Kabel et al., 2007). The removal of MeGlcA from glucuronoxylans is known to create such large portions of linear xylose units because they have block-wise substitution pattern as opposed to random or regular substitution, which is associated with arabinoxylans and arabinoglucuronoxylans (Kabel et al., 2007). Hence the glucuronoxylans were able to precipitate with relatively higher substitution than arabinoglucuronoxylans.

A variety of physical and chemical methods have been studied for reducing the degree substitution and solubility of xylans, the methods for selective removal of xylan substituents to allow controlled xylan precipitation are still not well developed (Schwinkal et al., 2006).

(a) Physical xylan modification

In physical modification of water soluble xylans, substituents are removed under sub or supercritical conditions (temperature from 40-170°C and pressure up to 150 bar), which leads to formation of spherical nano and microgels (Linder et al., 2003^{ab}; Gröndahl et al., 2004; Haimer et al., 2008). However, subjecting the xylan to such supercritical conditions, results in uncontrollable random cleavage of the side groups, which render such processes inefficient and inflexible for customised reduction of the solubility of the xylan. Furthermore, modifying the xylan at high pressure and temperature conditions might not permit simultaneous xylan modification and encapsulation or entrapment of heat sensitive bioactive agents and compounds in the modified xylan.

(b) Chemical xylan solubility reduction

Most of the chemical methods for reducing xylan solubility have been based on the reactions of xylans during kraft pulping (Sjöström, 1993, Henriksson and Gatenholm, 2002; Linder et al., 2003^{ab}; Köhnke et al., 2009). This is because the loss of side groups from the xylan main chain that occurs under the kraft pulping conditions, lead to xylan precipitation and adsorption on pulp fibres (Sjöström 1993). However, the chemical methods could result into an inefficient precipitation process because of being non-selective in degrading the xylans. Therefore, the chemical methods may offer limited control over the quality and quantity of the modified xylan. For example, under kraft pulping conditions, removal of up to 80% (from 10% to 2%) of side groups such as 4-*O*-methyl MeGlcA side chains was observed during the bulk delignification phase of the kraft cooking, where the temperature of the cook is about 160° and the alkalinity had decreased (Sjöström, 1993; Pinto et al., 2005; Danielsson, 2007). The loss of the substituents, such as acetyl, arabinose and MeGlcA side groups in kraft cook conditions causes the precipitation and aggregation behaviours of the xylan (Danielson, 2007), which is accompanied by loss of over 50% of the xylan (Sjöström, 1993).

Some of the chemical xylan modification methods that simulate the kraft pulping conditions to precipitate polymeric xylans have included longer treatments with oxalic acid, water, acetic acid and sulphur dioxide at high temperatures and alkalinity (100°C, pH 10.0, 120 min) (Walker, 1965; Westbye et al., 2006; Sternamalm et al., 2008). In other chemical modification processes, coacervation procedures, which are based on the principle of desolvation, using NaOH and neutralisation either, with concentrated HCL or acetic acid (Garcia et al., 2001; Ebringerová et al., 2005). Such chemical xylan modification procedures enhanced formation of xylan nano and microhydrogels (Garcia et al., 2001). Furthermore, precipitation of the xylan was achieved through oxidation methods (Ebringerová et al., 2005). The oxidation methods were based on the formation of crosslinkages between the xylan and either the ferulic or *p*- coumaric acid groups (Izydorczyk et al., 1991; Linder et al., 2003^b; Drechsler et al., 2008). Therefore, because of the association of these phenolic compounds with lignin, production of novel lignin-xylan nano-composites is possible.

Other chemical methods for reducing the hydrophilicity of water soluble xylans involved depolymerisation of the main xylan chain, which subsequently was crosslinked with other functional groups to induce the gelling behaviour. Examples of such derivatisation processes methods have included esterification, etherification, benzylation, hydroxypropylation, sulfation and emulsification reactions (Vincendon, 1998; Jain et al., 2001; Lindblad and Albertsson, 2005; Hettrich et al., 2006, Silva et al., 2007; Daus and Heinze, 2009). For example, Nagashima et al. (2008) used interfacial crosslinking polymerisation with different lipophilic phases to produce insoluble microcapsules hydrogels. Furthermore, the self assembling of xylan nano spheres (162-472 nm) for slow drug release was induced by esterification of the xylan with ibuprofen using carboxylic acid with N,N'-carbonyldiimidazole (CDI) that was sulphated by SO₃ and DMF (Daus and Heinze, 2009). In another study, production of elastic polysaccharide hydrogels was achieved through use of ionotropy effects between divalent cations and monovalent ions at room and moderate temperature (< 100°C) (Caruso et al., 2000; Schönberg et al., 2001; Wang et al., 2006) For instance, Köhnke et al. (2009) reduced the solubility of xylan through cationisation with 2,3-epoxypropyltrimethylammonium chloride (EPTMAC). Although the chemical modification methods have been successful in reducing solubility of the xylans, their use in applications where xylan purity is critical could be limited due to possible traces of residual chemicals in the products and problems of disposing the chemical discharges.

(c) Enzymatic modification of xylan solubility

Enzymatic methods are preferred to the physical and chemical methods for reducing solubility of water soluble xylans. This is because enzymes are specific for the structure of the substrate they react against (Van Beilen and Li, 2002). In addition, the enzymes that remove side chains from polymeric xylan do not stay bound to the substrate they attack (Biely, 2003). Such types of enzymes are recyclable (Van Beilen and Li, 2002) and therefore allowing multiple applications of the enzyme in xylan hydrolysis, which could reduce the cost of enzyme production and application. Furthermore, such enzymes could be considered non-contaminating to the formed products compared to the physical and chemical methods. Therefore, the enzymatically modified xylans could easily find applications in pharmaceutical or

medical industries with minimal purification requirements. However, the use of enzymes for controlled reduction of solubility of polymeric xylans has not been studied systematically.

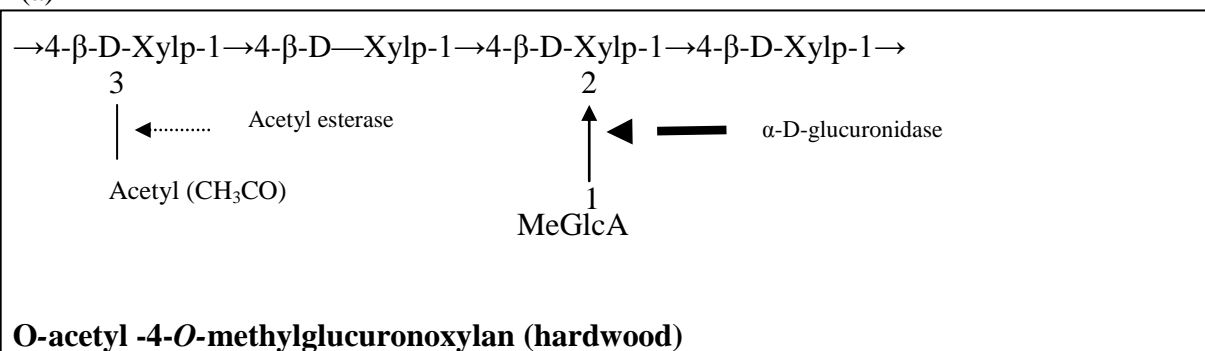
(d) Side chain removing enzymes

Based on the xylan structures (Fig. 1.1), the most important xylan side chain removing enzymes for modification of solubility properties of the xylan are the acetylsterases (EC 2.1.1.6), α -L-arabinofuranosidase (EC 3.2.1.55) and α -D-glucuronidase (EC 3.2.1.139/131), collectively referred to as accessory xylanolytic enzymes (Biely et al. 1992; De Vries and Visser, 2001; Tenkanen and Siika-aho, 2000; Zaide et al., 2001; Biely, 2003). The side chain removing enzymes from different or same microbial sources may differ in their substrate specificity (Biely, 2003). Detailed description of mode of action and preferred substrates of side chain removing enzymes from a wide variety of microorganisms has been compiled among others, by Eriksson et al. (1990), Viikari et al. (1993) and Biely (2003).

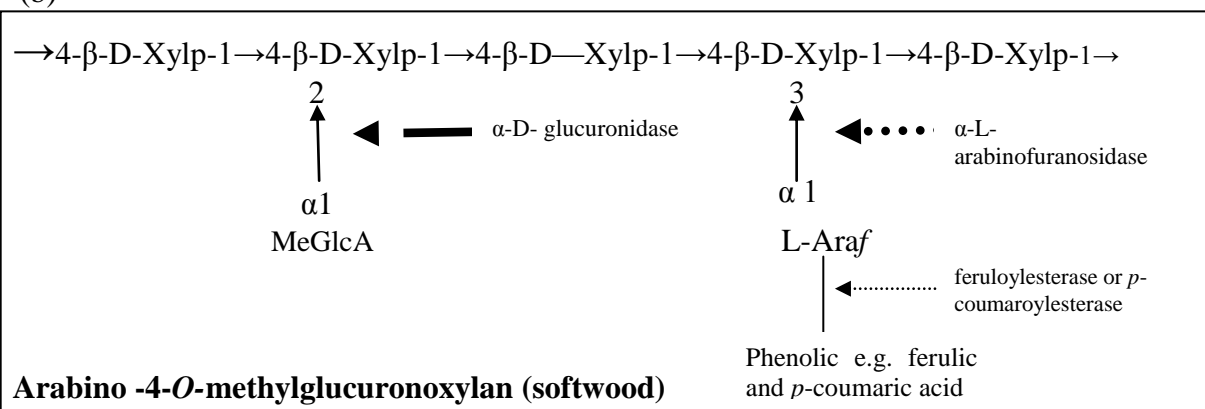
The side chain removing enzymes have special preferences for structural arrangement of the xylan main chain, and the position and frequency of the side group to be removed (Biely, 2003). Essentially, there are two main categories of side chain removing enzymes, which include those that act on both polymeric and oligomeric xylans and those that act strictly on branched or substituted oligosaccharides generated by main chain degrading xylanases (Rombouts et al., 1988). Enzymes responsible for reducing solubility of the xylans are mainly those that selectively remove the side chains from polymeric xylans (Biely, 2003). The side chain removing enzymes with polymeric xylan substrate specificity can permit customised precipitation of the xylan, which subsequently would provide options for production of higher value products compared to the current xylan utilisation (Numan and Bohsle, 2006).

The side chain removing enzymes have so far been used mainly to perform a supporting role to the xylan main chain degrading enzymes such as *endo*- β -1,4 xylanase (EC 3.2.1.8) and β -xylosidase (EC 3.2.1.37) during complete hydrolysis of

(a)



(b)



(c)

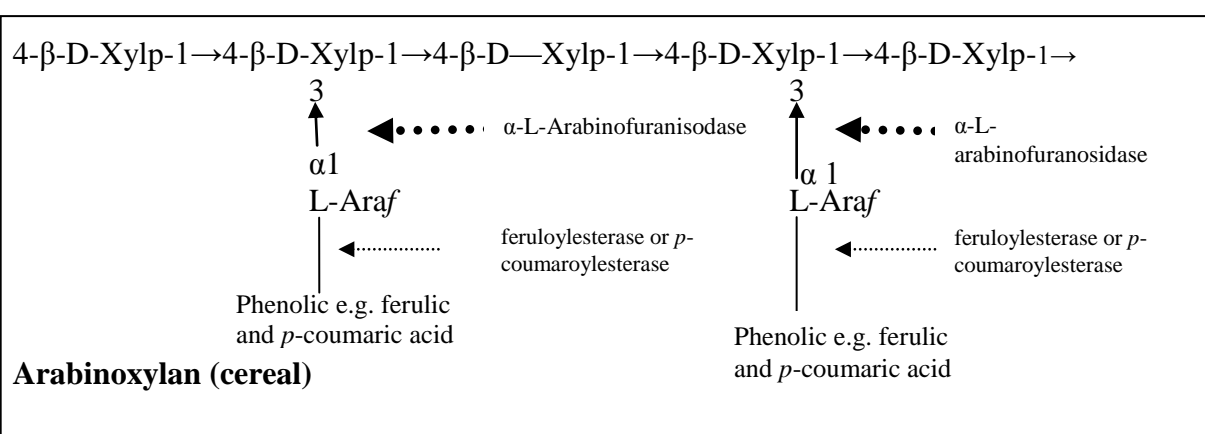


Figure 1.1: Illustration of structures of xylans from (a) hardwood, (b) softwood and (c) cereals. Arrows are showing the catalytic sites for the respective side chain removing enzymes. The xylan from hardwood (a) is called O-acetyl-4-O-methylglucuronoxylan, from softwood (b) is called arabino-4-O-methylglucuronoxylan and (c) from cereals is called arabinoxylan. Side chain removing enzymes include α-glucuronidase for removal of MeGlcA, α-arabinofuranosidase for removal of arabinose and esterases for removal of acetyl,

ferulic and *p*-coumaric acid groups from the xylan main chain consisting of 1, 4- β -D-linked xylose groups (Xylp).

the xylan (Kormelink and Voragen, 1993; De Vries and Visser, 2001; Koseki et al., 2003; Polizeli et al., 2005). Such synergistic actions have been investigated by several workers including Kormelink and Voragen (1993) and Rahman et al. (2003^{ab}) for production of the pulp and paper with improved properties (Kirk and Jeffries, 1996). For example, the xylan side chain removing enzymes were used together with xylanases to facilitate removal of residual lignin in bio-bleaching, to modify surface properties of thermo-mechanical pulps and to facilitate beating of pulp fibres (Puls et al., 1990; Kirk and Jeffries, 1996; Kuhad et al., 1997; Clarke et al., 2000; Viikari, 2003). In addition, side chain removing enzymes have been used in wine making to release phenolic compounds that improved aroma (Sánchez-Torres et al., 1996). However, side chain removing enzymes of technological importance in modification of solubility properties for production of insoluble hydrogels would be those that are capable of removing the side chains from polymeric xylans without requiring prior action of the xylan main chain degrading enzymes (Kaneko et al., 1998).

So far there are a few side chain removing enzymes with ability to cleave off side chains from polymeric xylans (Puls, 1992; Hazlewood, 1993; Gilead and Shoham, 1995; De Vries and Visser, 2001; Nagy et al. 2002; Shallom and Shoham, 2003). In addition, heteroxylan that contain more than one type of side chain or groups would require a combination of the side chain removing enzymes with different substrate specificities. The individual or synergistic application of such side chain removing enzymes would create opportunity for obtaining xylan of different substitution patterns that can be tailor made for specific degree of xylan precipitation.

Acetylesterases

Acetylesterases of *Streptomyces lividans* remove acetyl groups positioned at 2 or 3 on the xylose residue in acetylated hardwood xylans (Biely, 2003). Acetyl-4-*O*-methylglucuronoxylans from hardwood are water soluble from the high number and types of substituents (Puls and Schuseil, 1993). De-esterification (removal of the acetyl groups or removal of 4-*O*-methyl-D-glucuronic acid residues) led to decreased solubility of the xylan due to saponification of acetyl groups and formation of xylan

aggregates (Puls and Schuseil, 1993; Kabel et al., 2007) and stabilising effect on the neighbouring xylosidic bonds (Zaide et al 2001). Therefore, the removal of the acetyl groups could create new sites on the xylose units that can enhance functionalisation of the xylans. A variety of microorganisms, which include *Aspergillus*, *Penicillium*, *Schizophyllum* and *Trichoderma species*, produce acetyl esterase that belong to esterase family 1 and 5 as part of the enzyme consortium for complete degradation of the xylan (Viikari et al., 1993; Biely, 2003).

(i) α -L- Arabinofuranosidases

α -L-Arabinofuranosidases are well recognised enzymes for modification of xylan functional properties (Saha, 2000; Numan and Bhosle, 2006). The molecular, physico-chemical characteristics, substrate specificity and industrial applications of the arabinofuranosidases have been extensively reviewed (Saha, 2000, Numan and Bohsle, 2006). The arabinofuranosidases remove arabinose side groups from xylans by breaking the α -1,2-, α -1,3- and α -1,5- linked arabinose groups (Biely, 2003). However, the biocatalytic efficiency of the arabinofuranosidases on the arabinose–xylose linkages depends on the microbial source (Biely, 2003; Numan and Bohsle, 2006). Up to five glycoside hydrolase (GH) families of arabinose removing enzymes, which include GH 3, 43, 51, 54 and 62 have been identified according to amino acids composition (Tagawa and Kaji, 1988; Gielkens et al., 1997; Saha, 2000; Miyanaga et al., 2006). Alternatively, the α -L-arabinofuranosidases have been classified according to the substrate specificity, which allocates them into three groups. The first group are the arabinofuranosidases that act on both polymeric and xylooligosaccharides; the second group includes those that hydrolyse arabinose groups only from oligomeric xylans and third group, those that act on arabinoxylans only (Kaji, 1984; Beldman et al., 1993; Beldman et al., 1997; Saha, 2000; Biely, 2003). The family 51 arabinofuranosidases are active on *p*-nitrophenyl-arabinofuranoside (*p*NPA) and oligomeric xylans (Beylot et al., 2001). According to Biely (2003), the family 51 arabinofuranosidases are mostly arabinan rather than arabinoxylan degrading enzymes. This is because such enzymes have shown relatively higher preference for hydrolysing α -1,5 linkage of linear arabinan than α -1,2, α -1 and α -1,3 linkages of branched arabinoxylans. Family 62 arabinofuranosidases mostly attack α -1,2, α -1,3 linked arabinose side chains and α -L-arabinan residues of the main xylan chain (Komerlink et al., 1993, Wood and McCrae et al., 1996). Arabinofuranosidases

belonging to Family 54 include mostly fungal arabinofuranosidases, which act on *p*NPA and both oligomeric and polymeric xylans (arabinoxylans and arabinoglucuronoxylans) (Biely, 2003). Therefore the family 54 arabinofuranosidases would be the preferred enzymes for the selective removal of arabinose from water soluble xylans leading to precipitation.

The arabinofuranosidases of family 54 have been isolated and purified from a wide variety of the microbial sources including *Aspergillus* spp., *Aureobasidium pullulans*, *Penicillium purpurogenum* (Rombouts et al., 1988; Kaneko et al., 1995, Gilkens et al 1999, Saha, 2000; Rahman et al., 2003^{ab}). However, the most described arabinofuranosidase of family 54 belong to the *Aspergillus* spp. The arabinofuranosidase from *Aspergillus niger* (AbfB) is known to primarily act on terminal arabinose side chains releasing terminal α -1,2 and α -1,3 linked L-arabinofuranosyl residues from arabinoxylans (Tagawa and Kaji, 1988; Biely, 2003; De Wet et al., 2008).

(ii) *α -D-Glucuronidases*

α -D-Glucuronidases hydrolyse the α -1,2 glycosidic bonds between xylose and glucuronic acid side chains and its methyl derivative (Hazlewood and Gilbert, 1993; Nogawa et al. 1997; Tenkanen and Siika-aho, 2000; Zaide et al, 2001; Shallom and Shoham, 2004). The α -glucuronidases have been expressed and purified from a wide range of microorganisms (Zaide et al., 2001). It was observed that the α -glucuronidases, regardless of the microbial source, are known to be selective towards the structure of their substrate (Biely, 2003). Therefore, α -D-glucuronidase can be used for the removal of specifically positioned glucuronic side chains on the main xylan chain that would lead to customised reduction of xylan solubility. Most of the glucuronidases are capable of removing MeGlcA linked to a single xylopyranosyl residue or to a non-reducing terminal xylopyranosyl residue of xylooligosaccharides in aldouronic acids (Castanares et al., 1995; De Vries and Visser 2001; Biely, 2003). The glucuronidases that are active on xylan oligosaccharides as opposed to polymeric xylans are more effective when working in synergy with endoxylanases. For example, a combination of α -D-glucuronidase from *Phanerochaete chrysosporium*, endo (1 \rightarrow 4)- β -D-xylanase and α -L-arabinofuranosidase and β -D-xylosidase resulted in

87% release of the total MeGlcA in birch xylan compared to 66% in the presence of xylanase and β -xylosidase, 57% in the presence of xylanase and arabinofuranosidase and only 52% released with the mixture of α -4-*O*-methyl-D-glucuronidase and endo (1 \rightarrow 4)- β -D-xylanase (Castanares et al., 1995). Therefore, the α -D-glucuronidase with such substrate specificity would not be effective in reducing solubility of the xylans.

So far there have been a few fungal microbial systems identified to produce extracellular α -D-glucuronidase with polymeric xylan substrate specificity (Biely, 2003; De Wet and Prior, 2004; Tenkanen and Siika-aho, 2000; Khandke et al., 1989). Few fungal sources identified for producing α -D-glucuronidase (AguA) with polymeric glucuronoxylans and arabinoglucuronoxylans have included *Thermoascus aurantiacus* (Khandke, et al., 1989), *Schizophyllum commune* (Tenkanen and Siika-aho, 2000) and *Pichia stipitis* (Ryabova et al., 2009). Most of the AguA belong to the new GH family 115 (Kolenová et al., 2010). However, based on the amino acid sequence, the AguA from *Pichia stipitis* seems to belong to a different GH family (Ryabova et al., 2009).

(iii) *Microbial systems for production of xylan side chain removing enzymes*

The AguA and AbfB enzymes present a bio-based technology for selective removal of glucuronic and arabinose side chains respectively, which would allow custom modification of the solubility properties of the xylans. However, neither the AbfB nor the AguA are commercially available in sufficiently quantities of pure form and are difficult to isolate. Therefore, more efficient producing microbial systems are needed for selective over production of the AbfB and AguA enzymes (i.e. over production of AbfB or AguA free of xylan main chain degrading enzymes).

Although the AbfBs have been cloned in recombinant microbial systems, the extracellular production is still limited and therefore needs further improvement. On the other hand the recombinant production systems for extracellular production of AguA are yet to be developed. Among the microbial systems, *A. niger* is the commonly utilised microbial system for production of sufficient quantities of enzymes (gL^{-1}) of industrial importance (Wong and Saddler, 1993; Verdoes et al., 1995; Punt et al., 2002). However, the cost of producing pure enzymes for specialised applications constitutes a major proportion (>60%) of the cost of producing the

product (Hatti-Kaul and Mattiasson, 2001). The wild type strains of *A. niger* inherently secrete a wide variety of other extracellular enzymes including proteases and xylan main chain degrading enzymes such as endoxylanases and β -xylosidases in addition to the arabinofuranosidases and α -glucuronidase (Verdoes et al., 1995; Margolles-Clark et al., 1997; Punt et al., 2002). Consequently, the side chain removing enzymes have to be purified before utilising them for reducing solubility of the water soluble xylans, which increases the production cost (Hatti-Kaul and Mattiasson, 2001). For example, it takes two months to produce pure preparations of AguA from *S. commune* (Siika-aho, personal communication in 2008). Therefore, prevention of extracellular co-secretion of the xylan endoxylanases and proteases during production of the arabinofuranosidases and α -glucuronidase would advance their direct application in xylan precipitation. Prevention of co-expression of extracellular xylan main chain degrading enzymes and proteases is possible through manipulation of carbon and nitrogen composition of the cultivation medium respectively, and optimisation of process parameters, in particular by maintaining pH of cultivation media above acidic levels (Jarai and Buxton 1994; Van Den Hombergh et al., 1997; Margolles-Clark et al., 1997; Rose and Van Zyl 2002).

Low extracellular enzyme yields are often associated with inefficiencies in the transportation and secretion pathway, restrictiveness of the growing environment (medium, pH and temperature), hyperglycosylation, incorrect protein folding and proteolytic activity (Verdoes et al., 1995; Gouka et al., 1996; Van Den Hombergh et al., 1997). In recombinant systems, extracellular secretion of the enzymes is often limited at the transcription level depending on the site of integration, the number of introduced gene copies and the availability of trans-acting regulatory proteins (Verdoes et al., 1995; Gouka et al., 1997). Therefore, high quantities of the AbfB can be obtained by introducing strong promoters at transcriptional level (Gouka et al., 1997; Rose and Van Zyl 2002). Promoters for constitutive expression of recombinant enzymes in fungal systems include glyceraldehyde-3-phosphate dehydrogenase (*gpdp*) (Rose and Van Zyl 2002). Therefore the protease-deficient, non-acidifying pH mutant (*phmA*), *A. niger* D15 described by Wiebe et al. (2001) could enhance the extracellular production of the AbfB of *A. niger* and AguA of *S. commune*. The strain *A. niger* D15 has previously been used with great success in the production of both

heterologous and homologous enzymes (Rose and Van Zyl, 2002; Rose and Van Zyl, 2008; Alriksson et al., 2009; Van Zyl et al., 2009).

(e) Industrial application of xylan side chain removing enzymes

Although xylan–cellulose interaction is a natural phenomenon, such interactions can be customised to produce novel xylan-cellulose composites by controlled xylan modification. The key driving force in xylan-cellulose interactions as well as with other polymers is reduced xylan solubility, which is a function of molecular mass, degree of substitution, substitution pattern and surface charge (Ebringerová and Heinze, 2000). The xylan-cellulose interactions can be facilitated by application of AbfB and AguA possessing polymeric xylan substrate specificity. The utilisation of the AbfB and AguA for reducing solubility of xylan is a new area of research with novel applications that have not been extensively investigated. The lack of research in this area has been, in part, compounded by the limited availability of the side chain removing enzymes from polymeric xylan that are free of xylanase activity. So far studies on the application of the side chain removing enzymes to alter solubility of polymeric xylan have been based on α -L-arabinofuranosidase and acetylase (Tenkanen et al., 2007; Kabel et al., 2007; Höije et al., 2008). For example, oat spelt and wheat xylans were treated with α -L-arabinofuranosidase to selectively remove arabinose side groups for film formation with plasticisers (Tenkanen et al., 2007). In another study, Kabel et al. (2007) produced wheat arabinoxylans with structures altered with application of arabinofuranosidases that target different arabinose linkages attached to the xylose units prior to allowing the xylans to adsorb onto bacterial cellulosic materials. Enzymatic modification of the degree of xylan substitution was performed on prepared arabinoxylans from rye with arabinose to xylose substitution ratio of 0.50 to 0.20 by applying α -L-arabinofuranosidase possessing polymeric substrate specificity for formation of biofilms (Höije et al., 2008). Such studies created a foundation for developing enzymatic methods for selective removal of xylan substituents that would allow controlled reduction of solubility of xylan extracted from a wide range of feedstock.

Precipitated xylans are potential raw materials for production of hydrogels and biofilms, which inherently can swell to different degrees under varying solution conditions that could allow the xylans to be used as a biosensor (Kishida and Ikada,

2002; Numan and Bhosle, 2006). Furthermore, the less substituted xylans have increased affinity not only towards cellulose and lignin but to non-cellulosic materials as well. A study by Silva et al. (2007) investigated xylan as a coating material for sustained and targeted release of super para-magnetic particles in the human tract that are used in a medical scanning procedure known as magnetic resonance imaging (MRI). The xylan coating was used because of its chemo-stability in the physiological environment of the stomach and intestines (i.e. xylan is not degraded by enzymes found in the stomach but those found in the colon area) (Silva et al., 2007). In other studies, polymeric xylan was reacted with propylene oxide to form hydroxypropyl xylan that was further per-acetylated in formamide to form thermoplastic such as acetoxypopyl xylan thermoplastic composites, which are insoluble (Jain et al., 2001). Furthermore, Östernberg et al. (2001) showed that despite being negatively charged, the xylan were used as binding material for negatively charged mica surfaces. The interaction of the xylan and the mica surfaces was based on increased charge density of the xylan that led to more extended conformations on the mica, which resulted into long-range repulsions. Reis et al. (1992) indicated that negatively charged acidic xylans bound cationic gold to wall strips performed a similar function as a surfactant by generating an electrostatic repulsion between the microfibrils. Gabriellii et al. (2000) showed that cohesive forces existed between chitosan and xylan that resulted in crystallisation and electrostatic interactions between side groups of the xylan and amine group in the chitosan. Therefore, there is potential to increase the role of the side chain removing enzymes as well as of the xylans in lignocelluloses processing industries.

1.2 MOTIVATION

Globally, the pulp and paper industry is under pressure to reduce its impact on the environment while maintaining profitable margins. The industry is expected to reduce carbon footprint by implementing cleaner technologies that would promote sustainable use of resources, increase energy use efficiency, prevent pollution, and supply the market with products of high economic value. In addition, the current tougher global and national environmental regulations have increased demand for ecologically friendly products in place of fossil fuel derived products. There is a wide spectrum of lignocellulosic materials such as sugarcane bagasse, *E. grandis*, *P. patula* and *B.balcooa* and untapped supply of agricultural and forestry residues in South Africa from which xylans can be extracted. Therefore, the potential to establish secondary bio-economies on lignocelluloses is high. In addition, pre-extracting and modifying the xylans that would otherwise be wasted during processing of lignocellulosic materials during production of pulp and paper, timber and sugar products would ensure sustainable and optimal use of the renewable feedstocks. Furthermore, there is potential for reducing the energy use and chemical consumption when xylan extraction is integrated with pulping, which could make the pulp and paper making processes relatively cleaner and economically viable. This is because generation of organic and chemical waste could be reduced and at the same time, additional economic gains can be obtained from xylan bio-refining.

The advent of advanced biotechnology and bioprocess engineering has made it possible to develop recombinant microbial systems for over expressing enzymes that can be used for processing of lignocellulosic materials (wood, forestry products and agricultural residues) into high value products with less impact to the environment. Therefore, the enzyme technology being developed in this study is from a technical point of view a sustainable benign strategy for bringing higher level of process control and production of high value tailor-made biodegradable, non-toxic, biocompatible and renewable products with improved and new functionalities for use beyond the pulp and paper industry.

Chapter 2: Research approach

2.0 STUDY OBJECTIVES

The overall objective of the study was to develop an enzyme based technology for controlled reduction of xylan solubility and to demonstrate the technical performance of the insoluble xylan products in selected industrial applications. The specific objectives included:

1. Selective isolation and characterization of xylan from *Eucalyptus grandis*, *Pinus patula*, *Bambusa balcooa* and sugarcane (*Saccharum officinarum*) bagasse found in South Africa by using two selected mild alkali-low temperature isolation protocols.
2. Production and characterization of recombinant α -L-arabinofuranosidase (AbfB) with polymeric xylan substrate specificity expressed without xylanase activity in *A. niger* microbial system
3. Optimization of selective enzymatic hydrolysis for industrial application of isolated xylans, which included:
 - a. Assessing degree of selective removal of arabinose and 4-*O*- methyl glucuronic acid (MeGlcA) side groups by recombinant α -L-arabinofuranosidase (AbfB) of *Aspergillus niger* and purified α -D-glucuronidase of *Schizophyllum commune* (AguA) respectively from isolated xylan.
 - b. Identifying optimal levels of hydrolysis time, temperature and dosage of recombinant α -L-arabinofuranosidase and purified α -D-glucuronidase for maximum removal of arabinose and 4-*O*-methyl glucuronic acid side chains and the subsequent reduction in solubility of model xylans using response surface methodology.
 - c. Assessing the effect of individual and synergetic effects of recombinant α -L-arabinofuranosidase and purified α -D-glucuronidase on *in situ* modification and adsorption of xylans derived from wood and grass sources onto cellulosic material.

- d. Assessing the morphological features and ability of hydrogels produced from selective hydrolysis of xylans by recombinant α -L-arabinofuranosidase for encapsulation and slow release of horse radish peroxidase.

2.1 RESEARCH FRAMEWORK

The research activities were performed using a multidiscipline approach requiring expertise and knowledge from microbiology, wood science and chemistry and process engineering. The integration of the activities and the required expertise are depicted in Figure 2.1. The expertise from microbiology was relevant for construction of the microbial systems for expression of the side chain removing enzymes, whereas the knowledge from wood science and chemistry was necessary for characterisation of lignocellulosic materials for extraction of xylans. The production of the side chain removing enzymes, the enzymatic modification of the xylans and the investigation of the possible applications of the modified xylans constituted the process engineering component of the research. All activities were performed on the premise of developing an environmentally benign technology for production of biomaterials from renewable natural resources that have similar or improved functionalities compared to materials derived from fossil fuel.

2.2 SCIENTIFIC CONTRIBUTION

The planning, designing and execution of the experiments and interpretation of the results in this study were done by the author. Technical assistance was received from Dr S.H. Rose of Microbiology Department at Stellenbosch University in the cloning of the *abfB* gene in *A. niger* as specified in Chapter 4. The study has made scientific contributions in the following areas:

2.2.1 Selective isolation and characterisation of water soluble polymeric xylans from South African feedstocks (Chapter 3)

The study has generated information on the characteristics of South African feedstocks for extraction of unique xylans and for other lignocelluloses processes. In particular, the study has elucidated the structure and chemical composition of Eucalyptus (*Eucalyptus grandis*) and sugarcane (*Saccharum officinarum*) bagasse from Mpumalanga Province of South Africa and pine (*Pinus patula*) and bamboo (*Bambusa balcooa*) both from Western Cape Province of South Africa. In addition,

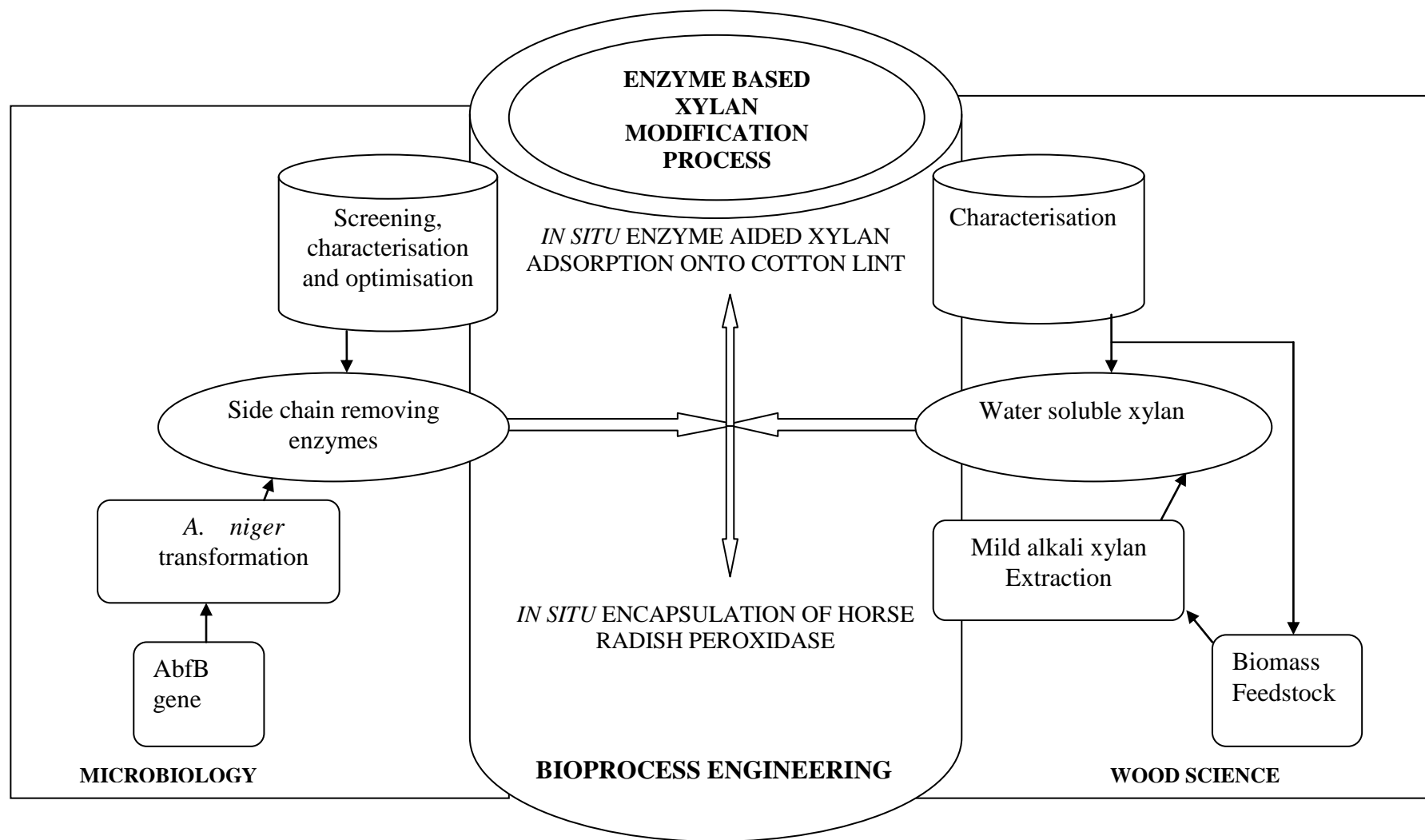


Figure 2.1: Research framework for the development of enzyme technology for reducing solubility properties of xylans leading to formation of speciality coating material and encapsulation matrix.

the extractability and chemical and structural features of the xylans extracted from the South African feedstocks were unveiled. Such information could be used for custom modification of the xylan properties to introduce different functionalities relevant for establishing a lignocelluloses biorefinery.

2.2.2 α -L-Arabinofuranosidase production in recombinant fungal system (Chapter 4)

An improved microbial production system and fermentation protocol for overexpressing recombinant AbfB in *A. niger* in high concentration and free of xylanase activity has been developed. The development of the recombinant *A. niger* production system is an advancement that would enhance production of speciality enzymes for novel applications in lignocelluloses processing, in particular, for xylan processing.

2.2.3 Enzymatic modification and industrial utilisation of xylans (Chapters 5-8)

The purified α -D-glucuronidase and the recombinant α -L-arabinofuranosidase (free of xylanase activities) are side chain degrading enzymes, which will advance lignocelluloses processing. The study has demonstrated novel ways of using unique group of α -D-glucuronidase for selective removal of MeGlcA from polymeric xylan. The recombinant α -L-arabinofuranosidase and purified α -D-glucuronidase were used as biological tools for reducing solubility of xylan extracted from the South African feedstocks in order to produce novel nanohydrogels. The xylan nanohydrogels can be used as biodegradable encapsulation, implantation matrix for slow delivery of compounds. In addition, the study has developed an improved enzyme based method for enhancing adsorption of water soluble xylans onto cellulosic materials thus allowing *in situ* modification (selective removal of side chains) and adsorption of the xylans onto cellulosic materials. Such technology has potential to transform and improve the efficiency of the wet end processes in pulp and paper making. Therefore, the study has novel contribution to advance research in the areas of green engineering, particle engineering, nanotechnology, biomedical engineering e.g. development of targeted and slow delivery systems and tissue replacement) and in surface chemistry.

Chapter 3: Selective isolation and characterisation of water soluble xylans from South African feedstocks using two mild alkali–low temperature extraction protocols

ABSTRACT

Eucalyptus grandis, *Pinus patula*, *Bambusa balcooa* (bamboo) and sugarcane (*Saccharum officinarum* L) bagasse (bagasse) found in South Africa were evaluated as sources of water soluble xylans for enzymatic modification. The xylan fractions were extracted from the feedstocks using two mild alkali-low temperature methods, one with ultrapurification fractionation denoted as Hoiye method and the other with ethanol precipitation denoted as the Lopez method. The chemical composition and structural characteristics of the feedstocks and the extracted xylans were determined using solid state ^{13}C CPMAS/NMR, ^1H and ^{13}C NMR and Fourier-transform infrared (FTIR) spectroscopy, high performance anion exchange chromatography (HPAEC) and colorimetric methods. The water soluble xylans were extracted from *P. patula*, bagasse, *E. grandis* and bamboo by the Hoiye method with extraction efficiencies of 71, 66, 35 and 20% respectively. In addition, the water soluble xylans were extracted from bagasse and *E. grandis* using the Lopez method with extraction efficiencies of 28 and 12% respectively. The xylans extracted from *P. patula*, bamboo and bagasse were identified as arabinoglucuronoxylans substituted with arabinose and MeGlcA side chains whereas those extracted from *E. grandis* were 4-*O*-methylyglucuronoxylan substituted mainly with MeGlcA and contained some acetyl groups. The xylans were water soluble and had degree of polymerisation (DP) of > 10. Therefore, the extracted xylans were in a form that would suit enzymatic modification. Furthermore, the integrity of the cellulolignin residue was preserved after xylan extraction, which could allow co-production of the xylans with other value added products in a biorefinery context.

3.0 INTRODUCTION

There is growing interest to utilise xylans as biodegradable polymers in similar ways as high molecular weight polysaccharides such as starch and cellulose. However, the xylans are not readily available in extracted form for industrial use (Ebringerová et al., 2005). Globally, there is adequate supply of feedstocks for extraction of the xylans. For instance, in South Africa, the potential supply of xylans is approximately 4.7 million metric tonnes (MT) of xylan per annum, which could be derived from fast growth species of Eucalyptus (*Eucalyptus grandis*), pine (*Pinus patula*) and giant bamboo (*Bambusa balcooa*) and from sugarcane (*Saccharum officinarum* L) process residues (bagasse). Lignocellulosic materials of similar types contain about 40-50% cellulose, 18-35% lignin and 25-35% hemicelluloses (Timell, 1967; Kuhad et al., 1997). The major hemicelluloses in most lignocellulosic materials are xylans (Timell, 1967). In hardwood and some grasses (e.g. bamboo), the xylan constitutes up to 90% whereas, in softwoods, the xylan constitutes about 50% of the hemicelluloses (Ebringerová, 2006).

The xylans are heteropolysaccharides consisting of a $-(1\rightarrow4)-\beta$ -D- linked xylopyranosyl backbone, which often are substituted with 4-*O*-methyl glucuronic acid (MeGlcA) and/or arabinose, acetyl, ferulic and *p*-coumaric side groups depending on the source (Timell, 1967; Fengel and Wegener, 1989). These xylan substituents form links with other polysaccharides and lignin resulting into formation of lignin-carbohydrate complexes (LCCs) (Fengel and Wegener, 1989; Sjöström, 1993; Joseleau et al., 1994). The LCCs are of concern when selective separation of the chemical cell wall components is required (Ebringerová et al., 2005). Consequently, harsh conditions similar to those employed in kraft pulping are applied to break down the LCCs, which could result in xylan degradation of up to 50% (Sjöström, 1993). Therefore, an estimate of over 500 000 MT of xylan could be wasting in black liquor from the 10 million MT of the wood processed into pulp annually by the kraft pulp process at South African pulp and paper mills (DME, 2004).

There is little prospect for recovery of xylans wasted in the black liquor. Consequently, the xylans are combusted together with other degraded products during recovery of pulping chemicals for production of steam and electricity for the pulp

mills (Sjöström, 1993). The proportion of energy generation derived from the xylan component of the black liquor is of low economic value, earning approximately \$32/MT, compared to more than \$2000/MT that could potentially be earned if the xylan is converted to biomaterials (Henningen, 2006). The direct recovery of the xylan polymers from the black liquor is also not economical because expensive technologies are required to fractionate the pure xylan polymers from the waste streams highly contaminated with chemicals and other organic degraded products (Bills et al., 1970; Haun, 1970; Sjöström, 1993). Therefore, pre-extraction of the xylans from the lignocellulosic materials prior to pulping would spare the xylan from being degraded and wasted in the black liquor waste streams (Ragauskas et al., 2006).

Highly selective methods are required for pre-extraction of the xylans to ensure that the extraction is less damaging to the integrity of the xylans and the cellulolignin residue, which subsequently, could be processed into pulp. Co-production of xylans with pulp from the lignocelluloses, would lead to establishment of biorefineries, analogue to the petroleum refineries (Ragauskas et al., 2006). Establishment of lignocellulosic biorefineries in South Africa makes economic and environmental sense due to the large amounts of xylans wasted during lignocelluloses processing. The selective pre-extraction would provide the xylans with structural and molecular features that could allow custom modification, in particular, by special enzymes that selectively remove or introduce functional groups similar to enzymatic modification of xyloglucan by xyloglucan endo-transglycosylases reported by Brummer et al. (2004) and Zhou et al. (2006).

The extraction of xylans prior to processing of lignocelluloses is not a common practice and has not been done for lignocellulosic materials found in South Africa. Various studies have been conducted elsewhere on isolation of xylans from lignocellulosic materials originating using mild alkali methods (De Lopez et al, 1996; Gabrielli et al., 2000; Glasser et al., 2000; Höije et al., 2005), solvent extractions (organosolv pulping) (Pan et al., 2005; Höije et al., 2005; Xu et al., 2006), hydrothermal aquasolv (Garrote et al., 2004; Bobleter, 2005; Mossier et al., 2005; Vázquez et al., 2005; Benkő et al., 2007; Roos et al., 2009) and ionic solvents (ionic liquids) extractions (Roger and Seddon, 2003; Lee et al., 2009). However, the yields and purity of xylans extracted by most of these methods are low to substantiate large

scale enzymatic modifications. For instance, Roos et al. (2009) reported xylan yield from barley husk as low as 9% using both microwave irradiation and microwave steam pretreatment. On the other hand, xylans were extracted from lignocellulosic materials by using mild alkali-low temperature extraction protocols (i.e < 16% alkali charge and < 80°C reaction temperature) with yields of >50% (De Lopez et al., 1996; Höije et al., 2005). The mild alkali methods have been used mainly for extraction of xylans from lignocellulosic materials derived from agricultural residues such as barley husks and barley straw with xylan yields of up to 83 and 57% respectively (De Lopez et al., 1996; Höije et al., 2005) but less on wood feedstocks. The selectivity of the mild alkali-low temperature methods for extracting polymeric xylans in relatively higher yields and pure form make them attractive for production of xylans for enzymatic modification.

The focus of this study was to extract water soluble xylans from *E. grandis*, *P. patula* and *B. balcooa* and sugarcane (*S. officinarum* L) process residues (bagasse) found in South Africa with structural features and chemical composition that would suit enzymatic modification. In the study, the effectiveness of the mild alkali-low temperature methods described by Höije et al. (2005) and De Lopez et al. (1996) were assessed for extraction of xylans from the South African feedstock. The extraction protocols were assessed based on (1) xylan extraction efficiency, (2) degree of polymerization and substitution of the extracted xylans, (3) chemical composition of the extracted xylans, (4) purity of the extracted xylans and (5) the structural integrity and chemical composition of the cellulolignin residue post xylan extraction.

3.1 MATERIALS AND METHODS

3.1.1 Raw materials

Xylans were extracted from *E. grandis* supplied by the Transvaal Wattle Cooperatives (Piet Retief, Mpumalanga Province, South Africa), *P. patula* (pine) (Stellenbosch forest plantations, Western Cape Province, South Africa), giant bamboo (*B. balcooa*) (from Paarl, Western Cape Province, South Africa) and sugarcane (*S. officinarum* L) bagasse, a by-product from the sugar processing industry, generously donated by TSB sugar (Nkomazi region, Mpumalanga province, South Africa). Oat spelt xylan (Sigma, X-0627), birch xylan (Roth, #7500) and mild alkali extracted H₂O₂ bleached bagasse xylan generously donated by Prof. A.M.F. Milagres (University of São Paulo, Lorena, Brazil) were used as model xylans to fingerprint the extracted xylans. Analytical grade sugars including arabinose, rhaminose, galactose, glucose, mannose, xylose and glucuronic acid from Merck were used as reference materials for monomeric composition of the feedstocks and extracted xylans.

3.1.2 Preparation of raw materials

Chips derived from wood and bamboo were dried at 105 °C to moisture content (mc) of \approx 10% and subsequently conditioned to a relative humidity of 55% at 20°C for at least 24 h prior to size reduction by a Condux hammer-mill, a Retch and a Wiley laboratory mill. The ground chips were fractionated by sieving using stackable sieves (ASTM) of 850 μ m/20 mesh size, 425 μ m/40 mesh size and 250 μ m/60 mesh size with a lid and pan. The particulates that passed through 425 μ m/40 mesh size but retained on a 250 μ m/60 mesh sieve were collected for chemical composition analyses and those retained on the 425 μ m/40 mesh were used for xylan extraction.

3.1.3 Chemical composition of the feedstocks

(a) Determination of moisture content, extractives, Klason lignin and Seifert cellulose

Moisture content of the feedstock materials was determined following National Renewable Energy Laboratory Analytical Procedure (NREL LAP) (Hammes et al., 2005). The moisture content was calculated as a percent (%) on dry biomass weight.

Extractives were removed in two sequential steps, starting with cyclohexane/ethanol (2:1) followed by hot (boiling) water extraction using a soxhlet apparatus. Both extractions were done according to TAPPI Test Method T 264 om-88 (TAPPI 2002-2003) and NREL LAP methods (Sluiter et al, 2005). The extractives were quantified on moisture free basis. Analysis of Klason lignin (acid insoluble) of the feedstocks was done following the NREL LAP method for determination of structural carbohydrates and lignin in biomass (Sluiter et. al., 2006) and TAPPI test procedures T249 cm-85 (2002-2003). Seifert cellulose content was determined according to the analytical method outlined by Browning (1967) and Fengel and Wegener (1989). In the method, extractive free material weighing 1.1g oven dry (o.d) was treated with a mixture of acetyl acetone (6 mL), dioxane (2 mL) and 32% HCl (2 mL) in round bottom flasks that were incubated in a boiling water bath for 30 min. The treated samples were transferred quantitatively into pre-weighed sinter glass crucibles for vacuum filtration and washing. The residues were successively washed with 100 mL each, of methanol, cyclo-dioxane, warm water (80°C), methanol and diethyl ether and subsequently dried at 105°C for 2 h. The Seifert cellulose content was defined as the weight of the dried residue presented as a percentage of the extractive free material.

(b) Determination of monomeric sugar profile in lignocellulosic materials

Monomeric sugar composition of the acid hydrolysate was determined using the NREL LAP method specified for determination of structural carbohydrates and lignin in biomass (Sluiter et. al., 2006). The quantification of the sugar composition in the acid hydrolysate was done after storage at -20°C for at least 24 h after terminating the acid hydrolysis using high performance anion exchange chromatography coupled with pulsed amperometric detection (HPAEC-PAD (Dionex)). The HPAEC-PAD was equipped with a gradient pump GP 50, a CarbpacTM PA 10 (4 mm x 250 mm) column and electrochemical detector (ED40). The eluents were 250 mM NaOH and Milli-Q-water in a ratio of 1.5:98.5 at a flow rate of 1 mL min⁻¹. Notably, sodium acetate (1M NaOAc) eluent was added during acid sugars (glucuronic/ 4-*O*-methyl glucuronic acid) analysis. The samples were filtered on 0.22 µm pore size filters before analysis on HPAEC-PAD (Dionex). The concentration of the sugars was determined from standard plots of the respective analytical grade sugars (arabinose, rhaminose, galactose, glucose, mannose, xylose and glucuronic acid). The data acquisition and analyses were performed using an inbuilt PEAKNET software

package. The amount of sugar was presented as a percentage on the basis of oven dry weight of the biomass.

(c) Determination of pentosan and ash content

Pentosan content in the feedstocks was determined according to TAPPI standards methods for measuring pentosans in wood and pulp T223 cm-84 m (TAPPI 2002-2003). The xylan content was calculated from a standard plot prepared from xylose (analytical grade) as percentage of the oven dry biomass. Ash content was determined by thermo-gravimetric method. Lignocellulosic samples (0.5 g) were incinerated in a Muffle furnace at $575 \pm 25^{\circ}\text{C}$ for 4 h or until a constant weight was obtained. Ash content was calculated as a percentage of the initial oven dry (o.d.) biomass.

3.1.4 Mild alkali xylan extraction

Extraction of xylans from the feedstock was performed using the two mild alkali extraction protocols adopted from Höije et al. (2005) and De Lopez et al. (1996) denoted as the Hoiye and Lopez method respectively. The Höije et al. (2005) extraction protocol first step was pre-treatment with HCl whereby lignocellulose sample weighing 50 g (o.d) was pre-treated with 0.5 L of 0.05 M HCl for 16 h at room temperature to remove fats and waxes. At the end of the first step, distilled water (0.5 L) was added and the pH adjusted to pH 4.0 using glacial acetic acid. The second step was delignification, which was done with application of 11.0 g of sodium chlorite [NaClO_2 (80% purity)] at 75°C for 2 h after the delignification, the mixture was cooled and subsequently filtered. The residue was washed with distilled water to remove the lignin before commencing the subsequent step. The third step was xylan solubilisation with 0.5 L of 1 M sodium hydroxide (NaOH) containing 0.5% sodium borohydride (NaBH_4) at room temperature. The NaBH_4 was added to stabilize degradation of hexose sugars. The reaction proceeded at room temperature for 16 h and was stopped by neutralizing the xylan extract to pH 7.0 with 3.0 M HCL. The fourth step was to concentrate the xylan extract to a third of its initial volume using a rotary evaporator (Rotavapor Büchi R-124, Switzerland) under vacuum at 40°C followed by ultrapurification using membrane dialysis (molecular weight cut off (MWCO) 12-14 kDa, Spectra/Pro 2 membrane Spectrum). The membrane dialysis purification was performed at 4°C for at least 2 days before freeze drying (Fig. 3.1a).

The Lopez extraction protocol consisted of three main steps, which included combined xylan delignification and solubilisation. In the protocol a sample of lignocellulosic material, 150 g (o.d) was soaked in warm NaOH (60°C) at an alkali charge of 13% at liquid: biomass ratio of 3:1 for 2 h. The liquid portion of the reaction mixture was squeezed out and the residue washed with 600 mL warm water (60°C). The washing was repeated 3 times. The xylan extract was recovered in a 2 L flask and was centrifuged (7000 rpm) for 5 min. The solid fraction, which consisted mainly of precipitated pectins, cellulose fibres, starch and fats, was separated by filtration using Büchner funnel. The pH of the xylan extract was adjusted with glacial acetic acid to pH 5.0 followed by concentration as indicated described for the Hoiye method. Subsequently, the xylan extract was precipitated by addition of two volumes of ethanol (95%). The extract was left at 4 °C overnight to allow precipitation and sedimentation of the xylan fractions. The xylan precipitates were recovered by centrifuging at 7000 rpm for 10 min followed by washing with ethanol before freeze drying (Fig. 3.1b). In both the Hoiye and Lopez methods, xylan extraction was performed without prior removal of solvent and hot water extractives. The extraction efficiency was defined as the yield of xylan per theoretical content of pentosans in the material. Noteworthy, the Lopez method was applied for extraction of xylan from only *E. grandis* and bagasse feedstocks.

3.1.5 Structural characterisation

Structural properties of the extracted xylans and feedstock residues were analysed before and after xylan extraction using solid state ¹³C-Nuclear Magnetic

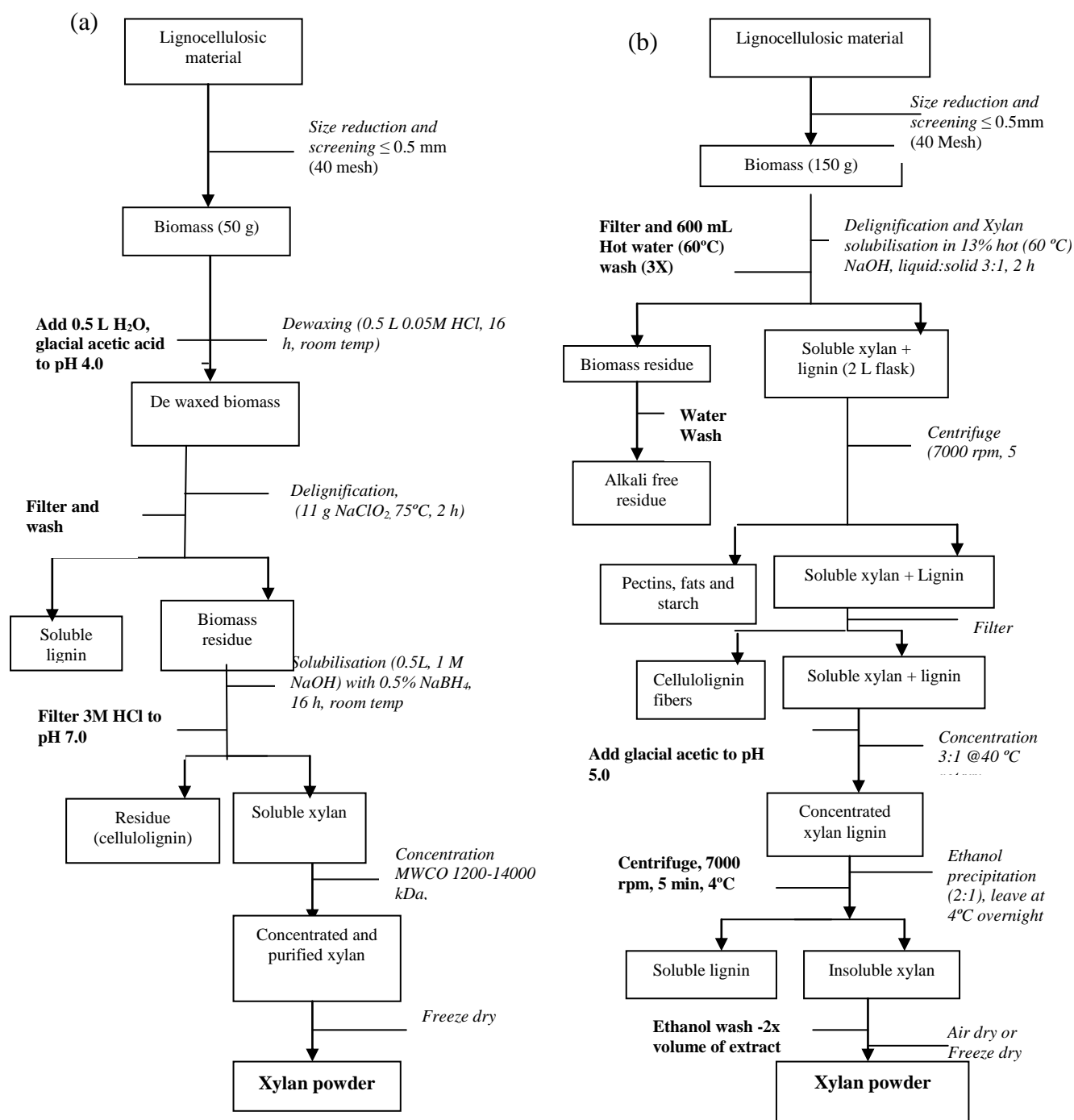


Figure 3.1: Process flow diagrams for mild alkali-low temperature water soluble xylan extraction protocols (a) Hoije and (b) Lopez xylan extraction protocols.

Resonance Cross-Polarisation/ Magic Angle Spinning (^{13}C -NMR CP/MAS), Liquid ^{13}C and 1H Nuclear Magnetic Resonance (^{13}C and 1H NMR) and Fourier Transform Infrared (FTIR) spectroscopy. In solid state ^{13}C -NMR CP/MAS, the samples were analysed on a Varian VNMRS 500 wide bore solid state NMR spectrometer with an operating frequency of 125 MHz for ^{13}C using a 6mm T3 probe at 25 °C. Dry samples

were loaded into 6 mm zirconium oxide rotors for analysis. Spectra were recorded using cross-polarisation and magic angle spinning (CP/MAS). The speed of rotation was 5 kHz, the proton 90° pulse was 5 μs, the contact pulse 1500 μs and the delay between repetitions 5 s. Chemical shifts were determined relative to Tetramethylsilane (TMS) by setting the downfield peak of an external adamantane reference to 38.3 parts per million (ppm). The carbon resonances in the solid state ^{13}C -NMR CP/MAS spectra were assigned according to Larsson et al. (1999), Renard and Jarvis (1999), Maunu (2002), Lahaye et al. (2003), Atalla and Isogai (2005), Virkki et al. (2005), Geng et al. (2006) and Oliveira et al. (2008).

In ^{13}C NMR spectroscopy, the samples were run either on a Varian Inova 400 or 600 NMR spectrometer. ^{13}C NMR spectra were collected with a 1.3s acquisition time and 1s pulse delay at 25°C. The ^{13}C spectra were collected overnight (minimum 19000 scans). The ^1H NMR spectra for the samples were collected after filtration with a 4.8 sec acquisition time at 50°C. ^1H spectra were collected with 64 scans and pre-saturation of the HDO peak. The ^{13}C and ^1H NMR spectra were interpreted according to assignment of characteristic signals of related feedstocks presented by Ebringerová et al. (1998); Vignon and Gey (1998), Renard and Jarvis (1999), Teleman et al. (2000) Teleman et al. (2002), Gröndahl et al. (2003), Lahaye et al. (2003), Sun et al. (2004^{ab}), Habibi and Vignon (2005), Pinto et al. (2005), Höije et al. (2005), Sims and Newman (2006); Geng et al (2006), Maunu (2008) and Shao et al. (2008).

In Fourier transform infrared (FTIR) spectroscopy, dry solid samples were recorded on a Nexus 670 spectrometer from Thermo Nicolet with the Smart Golden Gate attenuation total reflectance (ATR) accessory installed. This single-reflection accessory featured a diamond ATR crystal bonded to a tungsten carbide support equipped with ZnSe focusing lenses. The spectra were collected over the spectral range of 4000 to 650 cm^{-1} using 16 scans at 6 cm^{-1} resolution and were calibrated against a previously recorded background. Thermo Nicolet's OMNIC® Software was used for collecting and processing of the infrared spectra. The spectra signals for FTIR were interpreted according to characteristic bands presented by Fengel and Wegener (1989), Sun et al. (2004^{ab}), Xu et al. (2000), Sims and Newman (2006).

3.1.6 Estimation of xylan degree of polymerization (DP)

Degree of polymerization (DP) of the extracted xylan fractions was estimated on HPAEC (Dionex) using CarbopacTM PA100 column (4 x 250 mm) installed with a guard column and electrochemical detector (ED40) for pulsed amperometric detection (PAD). The CarbopacTM PA 100 column separates carbohydrate monomers and oligomers of up to DP 10, which usually elute within a retention time of 25 min (Combined CarboPacTM manual pp 52-56). Samples (10 μ L) were injected into the column in which they were eluted with helium degassed 0.25M NaOH, Milli-Q H₂O and 1 M NaOAc at a flow rate of 1 mL min⁻¹. Reference sugars and xylans used included arabinose, rhaminose, galactose, glucose, xylose, xylitol, mannose, birch xylan, oat spelt xylan and H₂O₂ bleached bagasse xylan. The presence of intense peaks of > 20 nC eluting within the 25 min retention time was an indication of presence of monomeric or oligomeric sugar with DP of <10 sugar units.

3.1.7 Determination of sugar profile of the extracted xylans

The sugar composition of the extracted xylan samples were determined on HPAEC–PAD (Dionex) on CarbopacTM PA10 column after mild acid hydrolysis according to Yang et al. (2005). Samples (0.1 g) were placed in Schott bottles (50 mL) into which 1 mL 72% H₂SO₄ was added. The mixture was incubated at 30°C in a water bath for 1 h. De-ionized water (30 mL) was added followed by autoclaving at 121°C for 1 h. The samples were cooled to room temperature before filtering. The filter cake was dried at 105°C for Klason lignin determination. The liquid fraction was filtered through a 0.22 μ m pore size filter before subjecting it to HPAEC–PAD (Dionex) on CarbopacTM PA 10 column. The monomeric sugars were quantified from standard plots of analytical grade arabinose, rhaminose, galactose, glucose, xylose and mannose. The total neutral sugar content of the samples was presented relative to the initial xylan oven dry (o.d) mass.

Uronic acid composition of the xylans and the feedstocks were quantified using both chromatographic and colorimetric methods. In chromatographic method, a two step acid hydrolysis method as described by Brienzo et al. (2009) was used. Xylan samples (150 mg, o.d mass) were hydrolysed in 0.75 mL of 72% (w/w) H₂SO₄ in McCartney bottles. The mixture was incubated at 45°C for 7 min in a water bath after which 22.5

mL of distilled water were added. The bottles were autoclaved at 121°C for 30 min. Upon cooling to room temperature, the liquid fraction was separated by vacuum filtering through glass micro fibre filters (GF/A- Whatman). The liquid fraction was further filtered through a 0.22 µm filter and kept frozen overnight at -20°C before analysing for MeGlcA content using HPAEC–PAD (Dionex) on Carbopac™ PA 10 column. Quantification of uronic acid was based on standard plots of GlcA (Sigma). Uronic acid losses during autoclaving were accounted for by autoclaved GlcA at 121°C for 1 h in 4% H₂SO₄. In colorimetric method, carbazole-sulfuric assay adopted from Li et al. (2007) was used. Total uronic acid concentration was determined from standard curve plot of D-galacturonic acid (Merck). The uronic acid content was presented as percentage of the initial amount of xylan.

3.1.8 Statistical Analysis

Statistical analysis with respect to calculating means and standard deviations were performed in Microsoft Excel 97-2003 version and STATISCA 2008 version.

3.2 RESULTS

3.2.1 Yield of extracted xylans

The chemical composition of *E. grandis*, bagasse, *P. patula* and bamboo feedstocks is presented in Figures 3.2a-c. The xylan content was determined as the pentosan content as shown in Figure 3.2c. Bagasse had the highest pentosans level of 22% (Fig. 3.2c). The pentosan content for *E. grandis* and bamboo was approximately 20% (Fig. 3.2c). The lowest pentosan level of 9% was found in *P. patula* (Fig. 3.2c). The xylans were extracted from *P. patula*, bagasse, *E. grandis* and bamboo by the Hoiye method with extraction efficiencies of 70, 65, 35 and 20% respectively (Fig. 3.2d). The extraction of xylans from bagasse and *E. grandis* using the Lopez method gave extraction efficiencies of 28 and 12% respectively (Fig. 3.2d).

3.2.2 Chemical and structural characteristics of xylans

(a) Monomeric sugar composition

The sugar profile of the xylans extracted using the Hoiye method from *E. grandis*, bamboo, bagasse and *P. Patula* contained of arabinose, galactose, glucose, rhaminose, xylose and uronic acid (Table 3.1). The xylose content of the xylans extracted from *E. grandis*, bamboo, bagasse and *P. Patula* by the Hoiye method was 82, 80, 71 and 61% respectively (Table 3.1). Whereas, xylose content of the model xylans, birch and oat spelt, were 80 and 87% respectively (Table 3.1). The arabinose content of bagasse, *P. patula* and bamboo xylan fractions extracted by Hoiye method was estimated to be 18, 16 and 11% respectively (Table 3.1). The *P. patula* xylan fractions had the highest glucose content of about 13% whereas that of *E. grandis* was 5% (Table 3.1). In addition, *E. grandis* contained galactose and some traces of rhaminose (1%) and arabinose (0.3%) (Table 3.1).

The total uronic acid content of the xylans extracted from *E. grandis*, bamboo and *P. Patula* by the Hoiye method was 12.80, 11.20 and 11.50% respectively, whereas, the xylan extracted from bagasse had the lowest uronic acids content of 8.50% (Table 3.1).

Table 3.1: Sugar composition of the extracted xylan fractions.

Xylan type	Total neutral sugar composition of xylan (%) ^a					Total uronic ^b acid (%) ^a	
	Arabinose	Galactose	Glucose	Rhaminose	Xylose	Raw material*	Xylan
Bagasse Hojie (BH)	17.4	3.4	8.0	-	71.0	6.0	8.5
Bamboo (BM)	10.5	2.9	7.1	-	79.5	6.7	11.2
<i>Eucalyptus grandis</i> Hoije (EH)	0.3	4.5	2.3	0.9	82.1	10.3	12.8
<i>Pinus patula</i>	15.5	10.0	13.2	-	61.3	8.7	11.5
Birch	-	0.4	19.5	-	80.0	-	10.0
Oat spelt** xylan	7.4	1.4	4.0	-	87.2	-	-

Note: ^a denotes mean of at least three samples with standard deviation < 0.1, ^b total uronic acids which may include glcA, 4-O-MeglcA and galactouronic acid, * percentage of extractive free raw material, ** Composition of oat spelt xylan (Sigma) is 10:75:15 for arabinose: xylose: glucose

(b) Acid insoluble lignin

The acid insoluble fractions (Klason lignin) of the xylan extracted by the Hoije and Lopez methods ranged from 16 to 55% (Fig. 3.3). The highest amount of acid insoluble residues (55%) was obtained from the xylan extracted from *P. patula*, whereas, the lowest (32%) was obtained from bagasse extracted by the Hoije method (Fig. 3.3). However, the acid insoluble residues obtained from xylan extracted from bagasse by the Hoije method were higher (28%) than those obtained from xylan extracted from bagasse by the Lopez method, which accounted for 16% (Fig. 3.3). In contrast, the acid insoluble residues obtained from *E. grandis* xylan extracted by the Hoije method was 31%, which was less than the acid insoluble residues of 38% obtained from *E. grandis* xylan extracted by the Lopez method (Fig. 3.3). However, the content of acid insoluble residues for the extracted xylan fractions from *E. grandis*, *P. patula*, bagasse and bamboo feedstocks by both the Hoije and Lopez methods were higher than the acid insoluble residues of 3.5% obtained from birch xylan (Fig. 3.3).

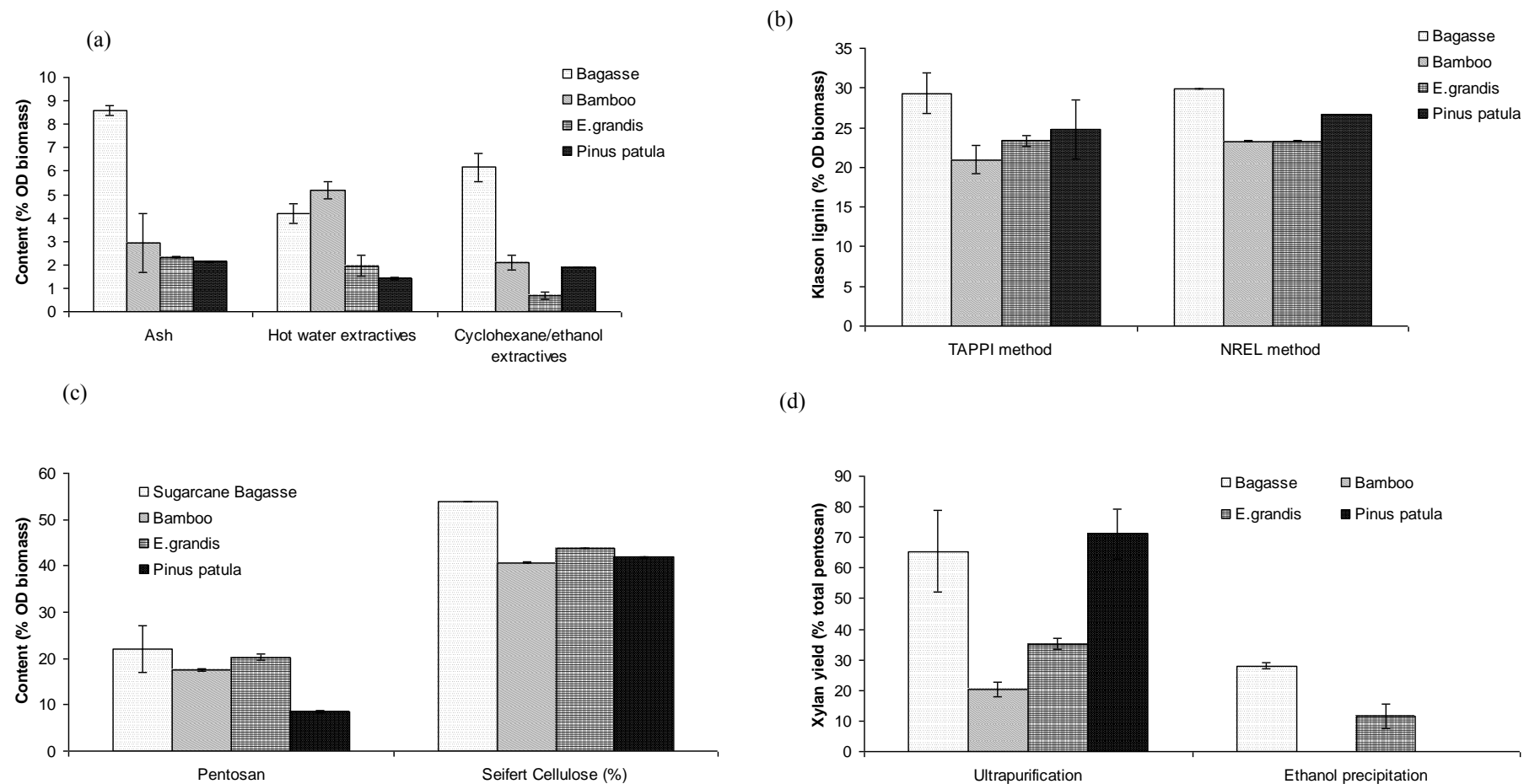


Figure 3.2: The composition of (a) extractives and ash, (b) Klason lignin, (c) cellulose and pentosan in bagasse, bamboo, *Eucalyptus grandis*, and *Pinus patula* and (d) xylan extracted with ultrapurification (Hoijs) and ethanol precipitation (Lopez) protocols. OD stands for oven dry.

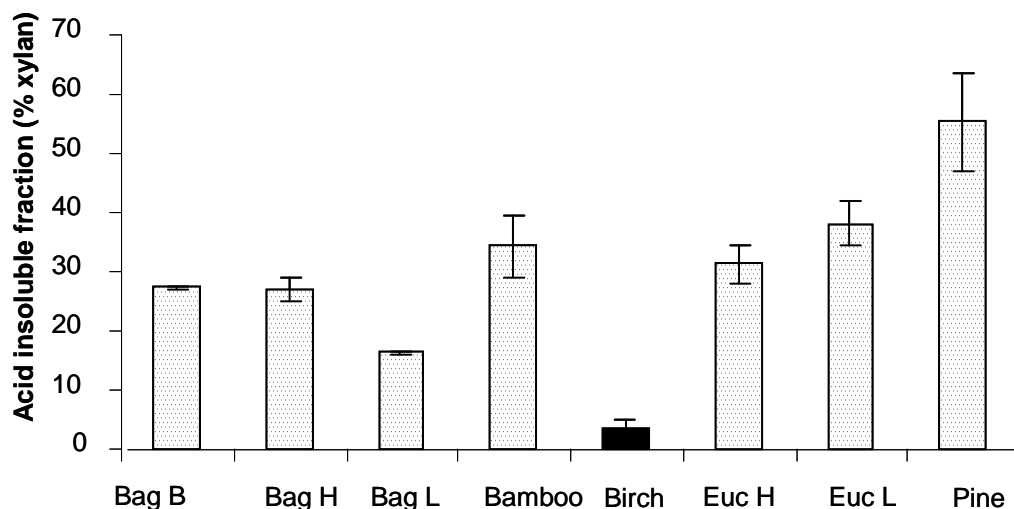


Figure 3.3: Insoluble fractions obtained after 72% H_2SO_4 acid hydrolysis of H_2O_2 bleached bagasse xylan (Bag B), bagasse xylan extracted by Hoije method (Bag H) and by the Lopez (Bag L), xylan extracted from bamboo, *E. grandis* by Hoije method (EU H) and by Lopez method (EU L), *P. patula* (Pine) and birch xylan (in black shed) .

(a) Physical characteristics of the extracted xylans

The xylans extracted by the two mild alkali-low temperature methods was less compacted and attained different pigmentations according to feedstock source and extraction method (Figures 3.4 a-f). The xylan fractions extracted by the Hoije method from bagasse (Fig. 3.4a) and *E. grandis* (Fig. 3.4d) were brighter in appearance than the corresponding xylan fractions extracted by the Lopez method shown in Figure 3.4b and Fig. 3.4e respectively. The colour of the xylan fractions extracted from bagasse feedstock by both Hoije and Lopez methods were darker than the xylan fractions extracted from *E. grandis*. Xylan fractions from bamboo had a green/yellowish pigmentation (Fig. 3.4c) whereas; the xylan fractions extracted from the *P. patula* by the Hoije method were the whitest of all the extracted xylan fractions (Fig. 3.4f).

(a) Estimation of degree of polymerisation

The elution profiles of the extracted xylan fractions were referenced to the elution profiles of the monomeric sugars (arabinose, rhaminose, galactose, glucose, xylose and mannose), xylitol sugar, birch xylan, oat spelt xylan and H_2O_2 bleached bagasse in order to estimate the degree of polymerisation (DP).



(a) Bagasse by
Hoije method

(b) Bagasse by
Lopez method

(c) Bamboo by
Hoije method

(d) *E. grandis* by
Hoije method

(e) *E. grandis* by
Lopez method

(f) *P. Patula* by
Hoije method

Figure 3.4: Xylan extracted from (a) and (b) bagasse, (c) bamboo, (d) and (e) *E. grandis* and (f) *P. patula* feedstocks using mild alkali–low temperature extraction methods adopted from Højje et al. (2005) denoted as Hoije method and from De Lopez et al. (1996) denoted as Lopez method.

The peaks of the reference monomeric sugars (Fig. 3.5a) including xylitol (Fig. 3.5b) eluted on CarboPacTM PA 100 column within a retention time of 5 min. The chromatogram for the reference xylans *vis* birch (Fig. 3.5c) and oat spelt (Fig. 3.5d) xylans, displayed peaks with a detector response of < 20 nC in the retention time between 3 and 6 min. In addition, the oat spelt xylan elution profile showed a high intensity peak with detector response > 300 nC between 0 and 3 min, which corresponded to retention time of xylitol (Fig. 3.5d). The chromatogram for H₂O₂ bleached bagasse xylan displayed multiple peaks of high intensities with detector response of over 100 nC within the retention time of 25 min (Fig. 3.6a) whereas, the chromatograms of bagasse xylan extracted by the Hoiije (Fig. 3.6b) and by the Lopez method (Fig. 3.6c) displayed low intensity peaks (<20 nC) within the retention time of 25 min. The peaks corresponding to the peak for xylitol were evident in the chromatogram of xylan extracted by the Hoiije method from *E. grandis* (Fig. 3.7a), bamboo (Fig. 3.7c) and *P. patula* (Fig. 3.7d).

The ¹H NMR and ¹³C NMR spectra of xylans extracted from bagasse (Figs. 3.9a and b), *E. grandis* (Figs. 3.9c and d), bamboo (Figs. 3.10a and b) and *P. patula* (Figs. 3.10c and d) were referenced to the ¹H NMR and ¹³C NMR spectra of xylan from birch (Fig. 3.8a and b), H₂O₂ bleached bagasse xylan (Fig. 3.8c and d) and oat spelt xylan (Fig. 3.8e and f). The ¹H NMR spectra of xylans extracted from bagasse (Figs. 3.9a), *E. grandis* (Figs. 3.9c), bamboo (Figs. 3.10a) and *P. patula* (Figs. 3.10c) displayed characteristic proton signals from xylose, 4-*O*-methyl-D-glucuronic acid (MeGlcA) and arabinose units at chemical shifts (δ) between 3.3 to 5.7 ppm. The signals appearing at δ 4.44/4.45, 3.50, 3.67 and 4.01 ppm that associate with H-1, H-3, H-4 and H-5 of the xylose units substituted with MeGlcA linked at O-2 (Vignon and Gey, 1998) appeared in the ¹H NMR spectra for xylans extracted from bagasse by the Lopez (Fig. 3.9a, spectra 1) and by the Hoiije method (Bag H) (Fig. 3.9a, spectra 2). In addition, the signals from proton resonances associated with H-1, H-2 and H-4 of D-xylopyranosyl units substituted with MeGlcA at O-2 and acetyl group at O-3 were displayed at δ 4.72., 3.76/3.75, 3.97/3.96 ppm (Fig. 3.9a, spectra 1&2).

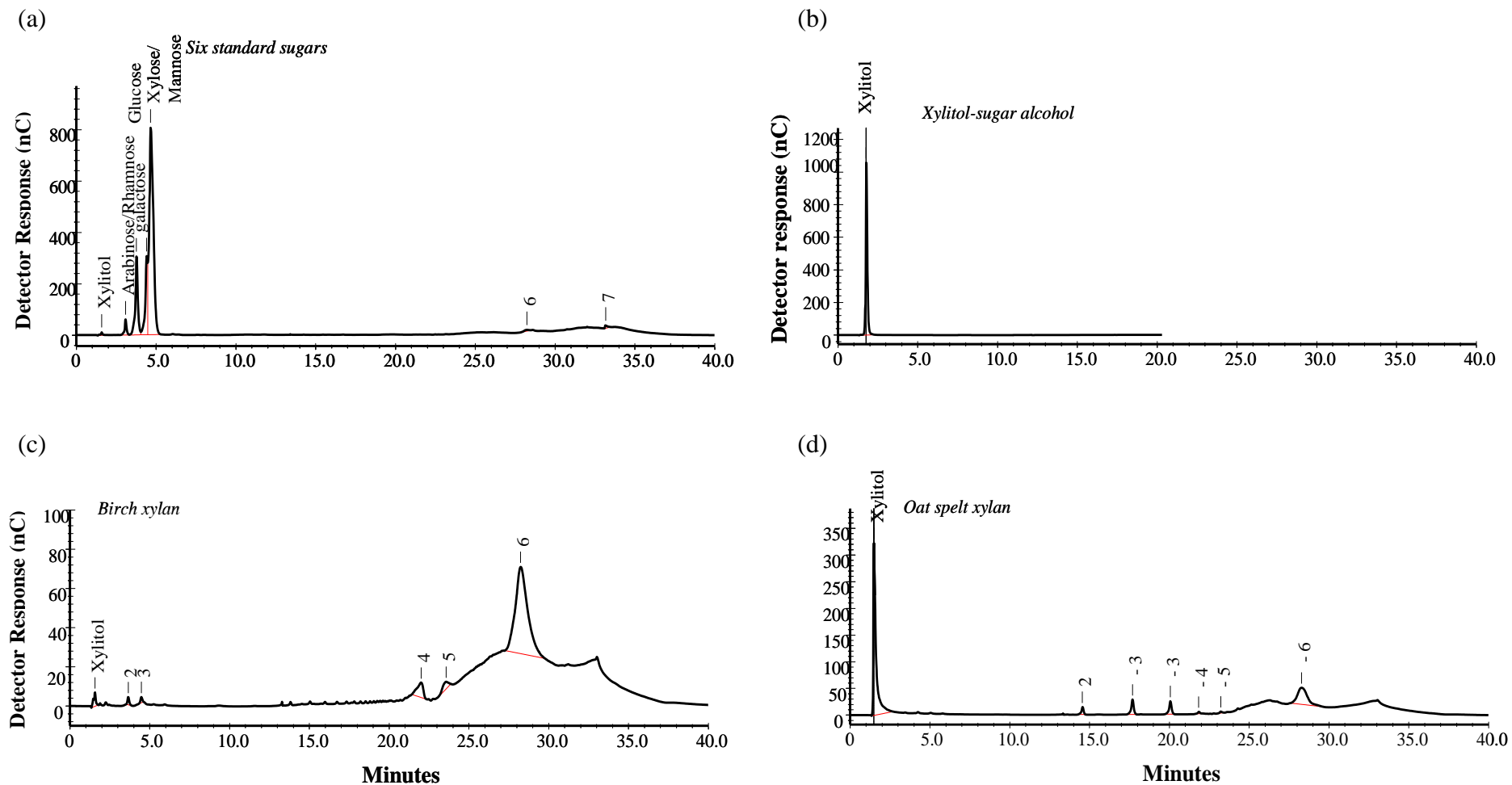


Figure 3.5: Elution profiles on HPAEC-PAD (Dionex) CarboPac™ P10 column of (a) monomeric sugars, (b) xylitol, (c) birch xylan (Roth) and (d) Oat spelt xylan.

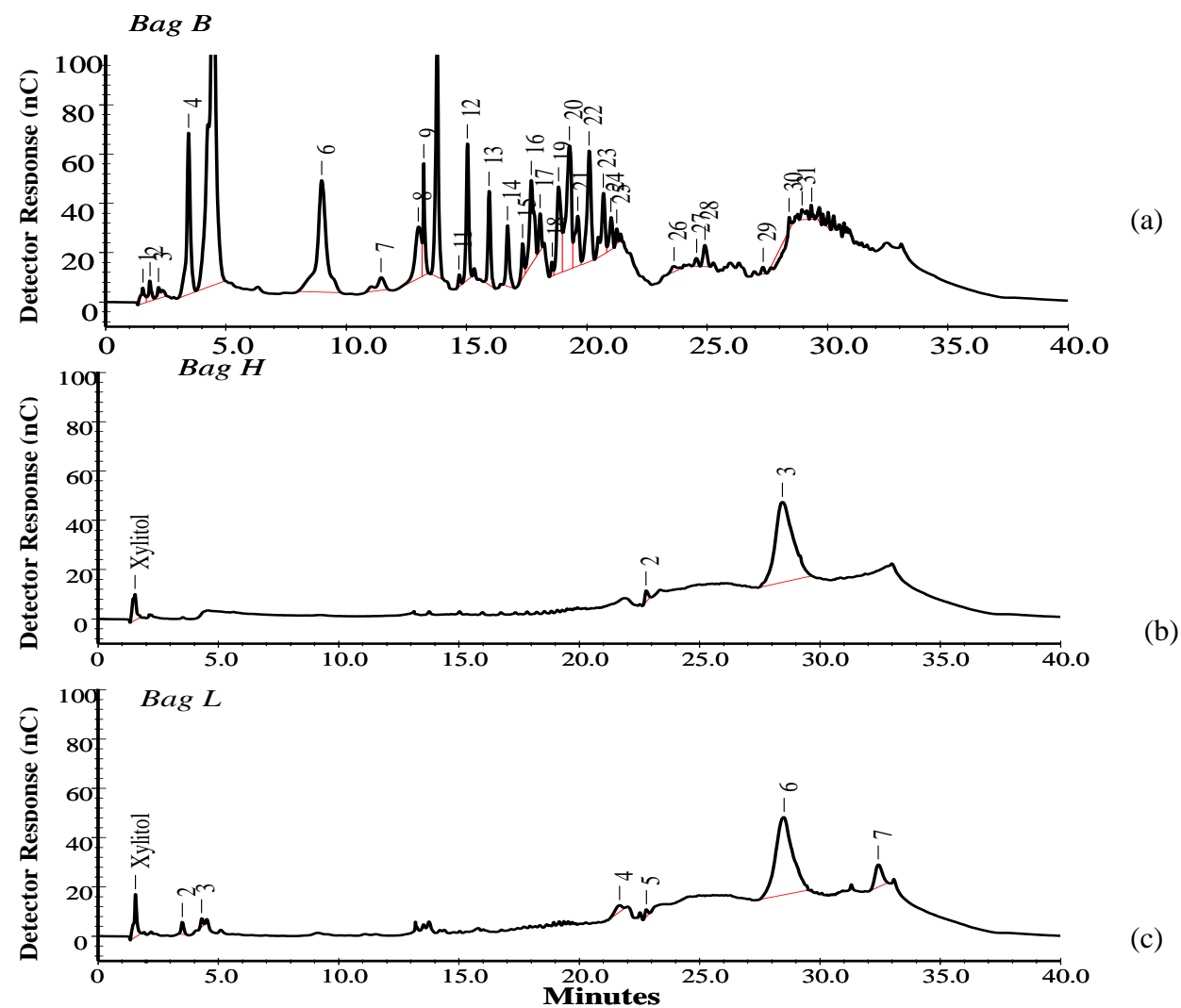


Figure 3.6: Elution profiles on HPAEC-PAD (Dionex) CarboPac™ column P10 of (a) H₂O₂ bleached bagasse xylan (Bag B), (b) bagasse xylan extracted by Hoiye method (Bag H) and (c) bagasse xylan extracted by the Lopez method (Bag L).

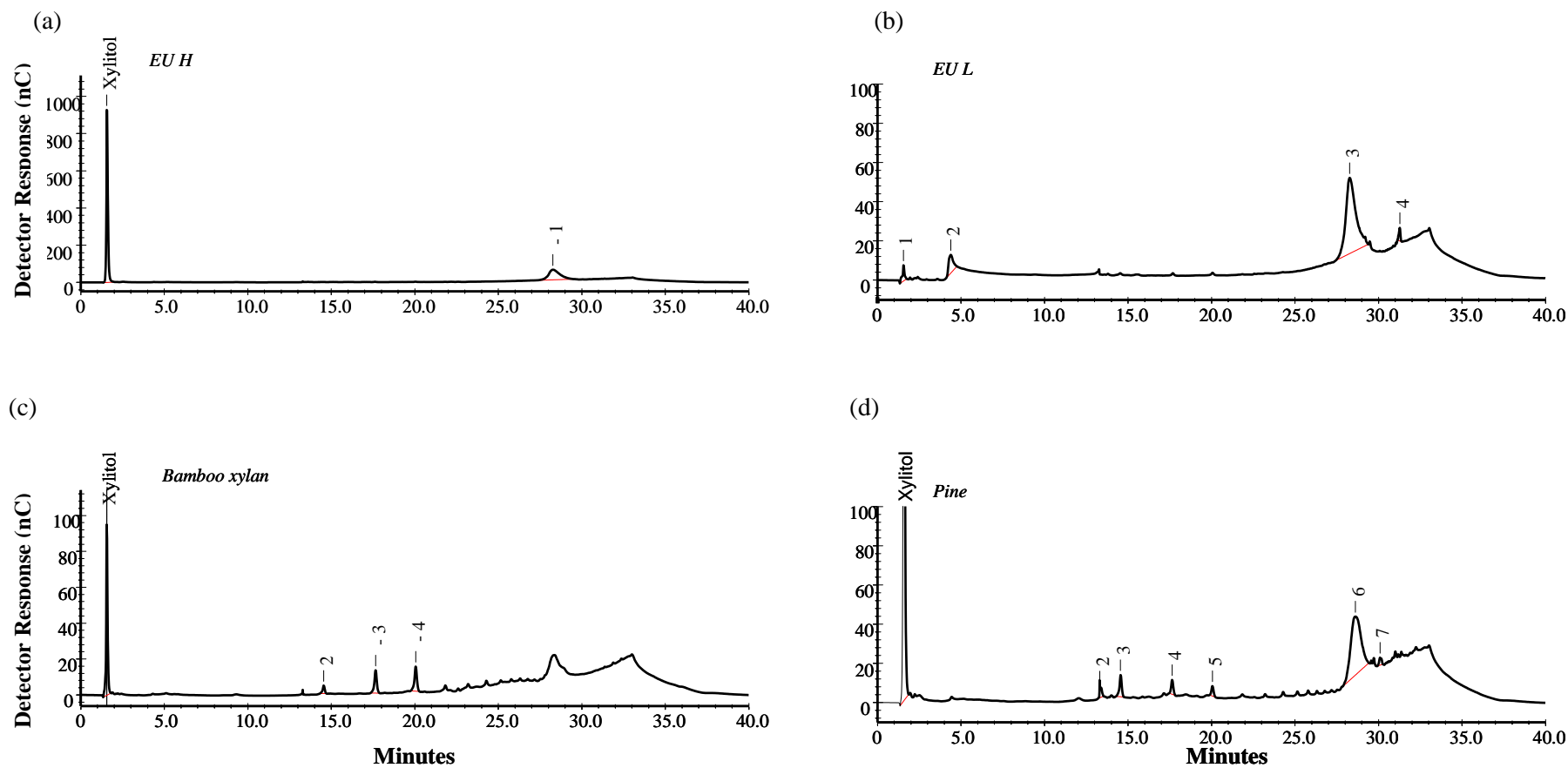


Figure 3.7: Elution profiles on HPAEC-PAD (Dionex) CarboPacTM column P10 of xylan fractions from (a) *E. grandis* by Hoije method [EU H], (b) *E. grandis* by Lopez method [EU L], (c) bamboo and (d) *Pinus patula*.

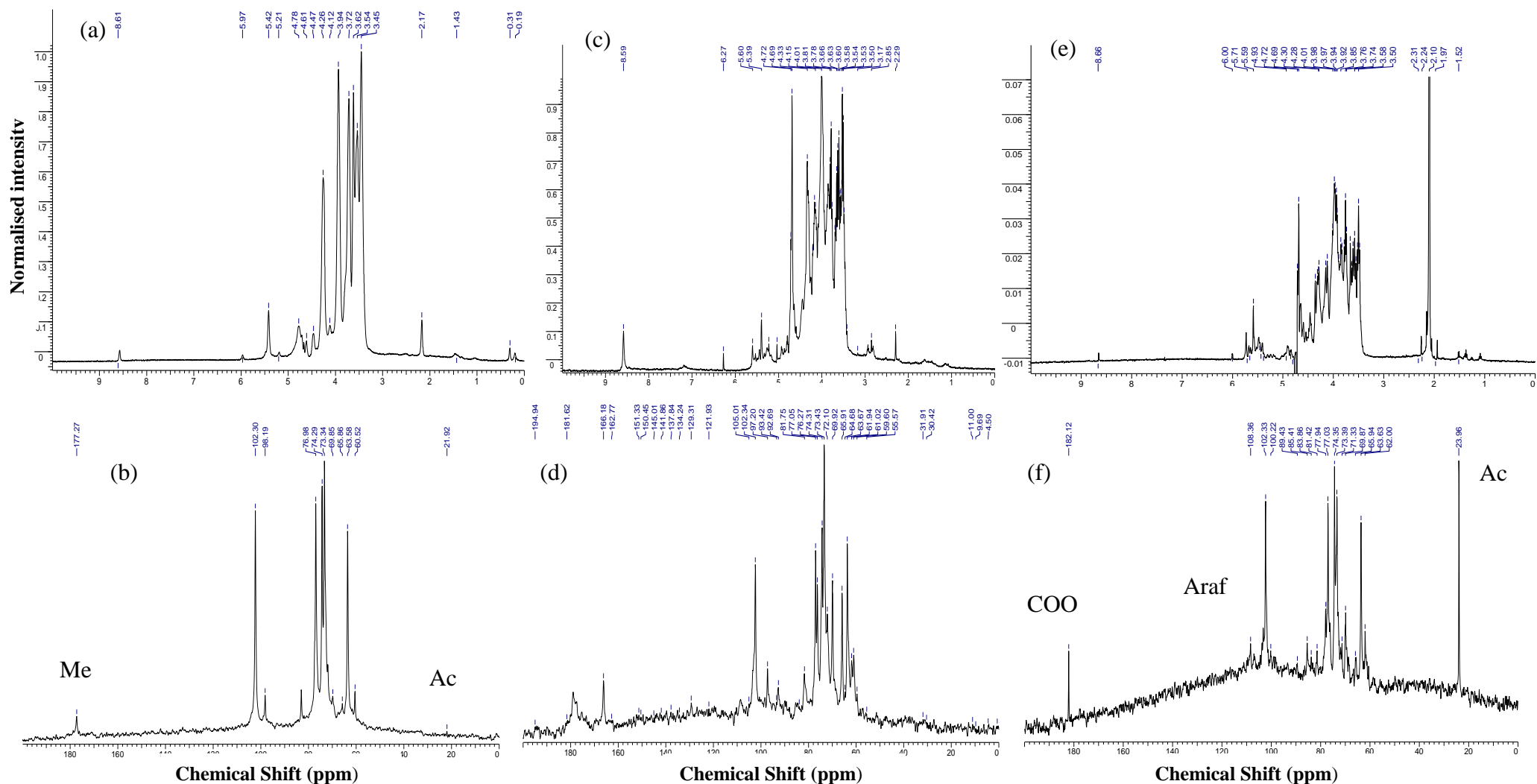


Figure 3.8: Characterisation of xylan by (a) ^1H -NMR and (b) ^{13}C -NMR analyses of birch xylan, (c) ^1H -NMR and (d) ^{13}C -NMR analyses of H_2O_2 bleached bagasse xylan (Bag B), and (e) ^1H -NMR and (f) ^{13}C -NMR analyses of oat spelt xylan. Me denotes Methyl group from MeGlcA, Ac = acetyl group, Ar = arabinose, COO = carbonyl group. The spectra were interpreted according to Vignon and Gey (1998), Sims and Newman (2006), Sun et al. (2004^{ab}), Ebringerová et al. (1998), Höije et al.(2005), Shao et al. (2008), Xu et al. (2000), and Habibi and Vignon (2005).

The ^1H NMR spectra of the xylans extracted from *E. grandis* using the Lopez method (Fig. 3.9c, spectra 1) and the Højje method (Fig. 3.9c, spectra 2) displayed signals for xylose units substituted with MeGlcA at δ 4.48, 3.96/3.99, 3.63/3.68 and 3.50/3.52 ppm. According to Sims and Newman (2006), such characteristic chemical shifts originate from D-xylopyranosyl units residues substituted with MeGlcA at O-2 and acetyl group at O-3. In the same spectra (Fig. 3.9c), the proton signals originating from H1 and H3 of the 4-*O*-methylMeGlcA residues were evident at δ 5.48/5.49/5.46 ppm and between 1.06 and 1.54 ppm δ 5.16 and 3.63 ppm. The signals for the MeGlcA residues, which were identified according to Sun et al. (2004^{ab}) and Ebringerová et al. (1998) appeared at 5.47/5.48 ppm in the proton spectra of xylan extracted from bamboo (Fig. 3.10a) and at δ 5.16 and 3.63 ppm in the proton spectra of xylan extracted from *P. patula* (Fig. 3.10 c). Furthermore, characteristic signals originating from C-2 linked arabinose to xylose units were displayed in the ^1H NMR spectra of the xylans extracted from bagasse (Fig. 3.9a), *E. grandis* (Fig. 3.9c), bamboo (Fig. 3.10a) and *P. patula* (Fig. 3.10c). In the proton spectra for xylan extracted from bagasse by Højje (Fig. 3.9a (ii)) and by Lopez (Fig. 3.9a (i)) methods, the C-2 linked arabinose were identified at δ 5.58, 5.60, 4.29/4.30 ppm, according to Højje et al. (2005), Ebringerová et al. (1998). The presence of arabinose in the proton spectra of xylan extracted from bamboo (Fig. 3.10a) and from *P. patula* (Fig. 3.10c) were identified inter alia, at δ 5.58/5.59 ppm and 5.47/5.48 ppm in accordance to Ebringerová et al. (1998) and Vignon and Gey (1998). Signals for arabinose residues were also present in the proton spectra of xylan extracted from *E. grandis* by the Lopez method (Fig. 3.9c (i)) and by the Højje method (Fig. 3.9c (ii)) between δ 3.83 and 3.85 ppm. The presence of *O*-2 linked acetyl groups were identified based on the descriptions by Ebringerová et al. (1998), Vignon and Gey (1998), Teleman et al. (2000), Teleman et al. (2002) at δ 4.4-5.5ppm. In addition, broad signals associated with aromatic or phenolic compounds originating from lignin residues (Højje et al., 2005; Xu et al., 2006; Shao et al., 2008; Oliveira et al., 2008) were located between δ 6.5 and 7.9 ppm in the proton spectra of the extracted xylan fractions (Figs. 3.8-10).

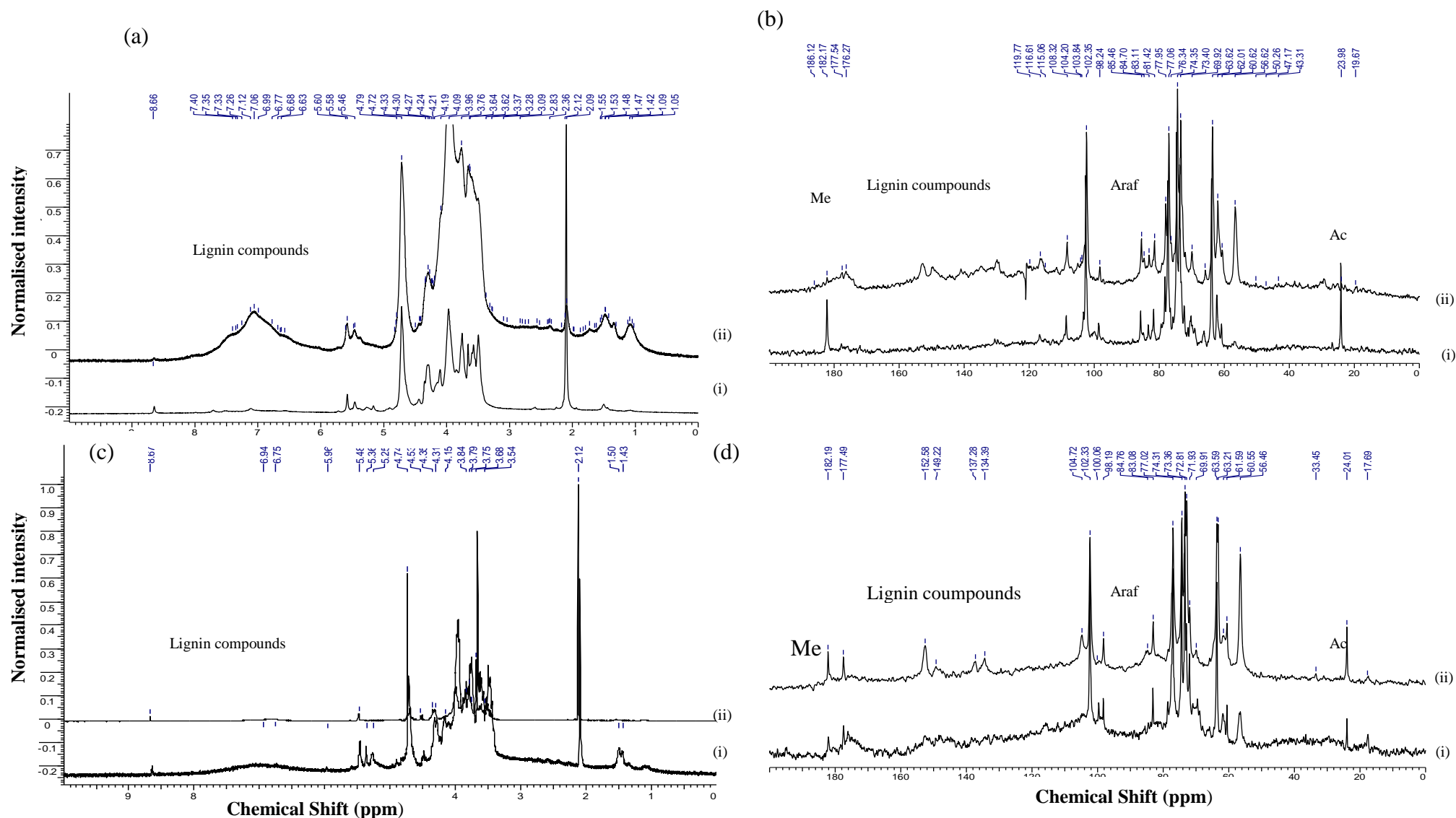


Figure 3.9: Characterisation by (a) ^1H -NMR and (b) ^{13}C -NMR analyses of xylan extracted from bagasse, c) ^1H -NMR and (d) ^{13}C -NMR analyses of xylan *E. grandis*: In the spectra (i) xylan extracted by the Lopez method (Bag L or EU L), and (ii) extracted by Højje method (Bag H or EU H). The spectra were interpreted according to Vignon and Gey (1998), Sims and Newman (2006), Sun et al. (2004^{ab}), Ebringerová et al. (1998), Højje et al. (2005), Shao et al. (2008), Xu et al. (2000), and Habibi and Vignon (2005).

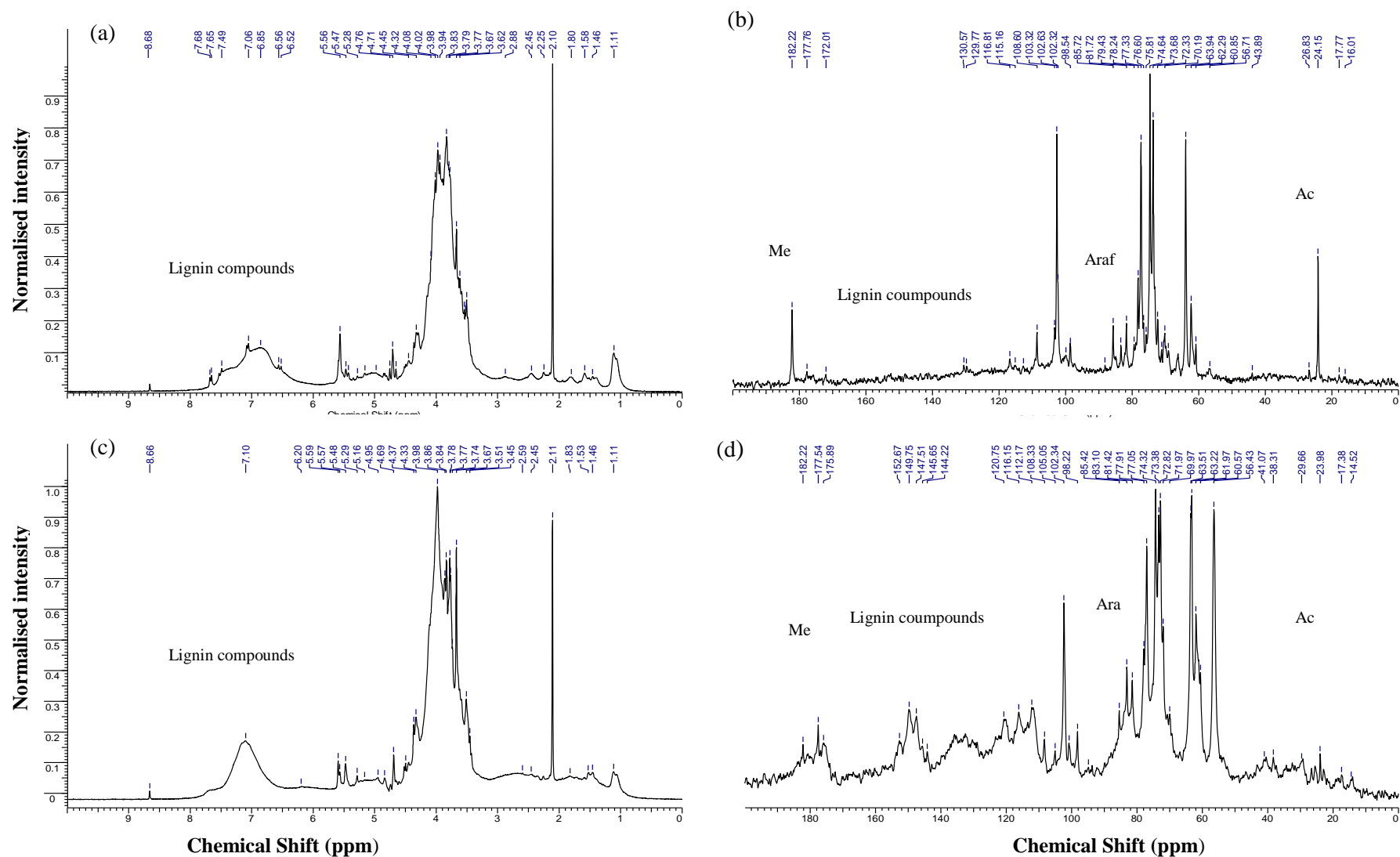


Figure 3.10: Characterisation of xylan by (a) ^1H -NMR and (b) ^{13}C -NMR analyses of bamboo, (c) ^1H -NMR and (d) ^{13}C -NMR analyses of *P. patula*. The spectra were interpreted according to Vignon and Gey (1998), Sims and Newman (2006), Sun et al. (2004^{ab}), Ebringerová et al. (1998), Höije et al. (2005), Shao et al. (2008), Xu et al. (2000), and Habibi and Vignon (2005).

In the ^{13}C NMR spectra, the characteristic carbon resonances from the five carbons of (1 \rightarrow 4) linked β -D- xylopyranosyl residues derived from bagasse (Fig. 3.9b), *E. grandis* (Fig. 3.9d), bamboo (Fig. 3.10b) and *P. patula* (Fig. 3.10d) were reflected between δ 103 and 62 ppm. The presence of MeGlcA residues in the extracted xylan fractions was evident between δ 97 and 100 ppm, 83 and 84 ppm, 172 and 179 ppm and 59 and 61 ppm, which originate from characteristic C-1, C-4, C-6 and C-5 carbon resonances respectively (Ebringerová et al., 1998; Xu et al., 2000; Habibi and Vignon, 2005). In the ^{13}C NMR spectra for *E. grandis* xylan fractions (Fig. 3.9d), the resonance originating from C-1 of xylose units with C-2-linked arabinose groups were displayed at $\delta \approx 102.33$ ppm. The carbon resonances originating from the C-1, C-2, C-3, C-4 and C-5 of the xylans extracted from bagasse by the Lopez method are shown in Figure 3.9b (i) and by the Hoiye method in Figure 3.9b (i). The signals for carbon resonances originating from arabinose residues of xylans extracted from bagasse, bamboo and *P. patula* were in the ^{13}C NMR spectra displayed at δ 112.50/ 111.56 and 89.31 ppm (Figs. 3.9b, 3.10b and 3.10d), which according to Vignon and Gey (1998) correspond to C-1 and C-2 of C-2 linked arabinose residues respectively. The C-1, C-2, C-4 resonances belonging to arabinofuranosyl residues mono substituted xylose units at O-3 (Ebringerová et al., 1998) were present in the ^{13}C NMR spectra of xylans extracted from bamboo (Fig. 3.10b) and *P. patula* (Fig. 3.10d) at δ 108.60/108.33 ppm, 81.71/81.42 ppm, 85.72/85.43 ppm respectively. The signals indicating presence of acetyl, phenolic and aromatic groups arising from lignin compounds and the presence of hexose sugars were identified in the ^{13}C NMR spectra of xylans extracted from bagasse (Fig. 3.9b), *E. grandis* (Fig. 3.9d), bamboo (Fig. 3.10b) and *P. patula* (Fig. 3.10d) between δ 21-24 ppm. However, such signals were absent in the ^{13}C NMR spectra of birch xylan (Fig. 3.8b) and H_2O_2 bleached bagasse xylan (Fig. 3.8f). The methoxyl groups signifying presence of lignin compounds (Ebringerová et al., 1998; Sun et al., 2005; Xu et al., 2006; Maunu 2008) were identified in the ^{13}C NMR spectra of xylans extracted from bagasse (Fig. 3.9b), *E. grandis* (Fig. 3.9d), bamboo (Fig. 3.10b) and *P. patula* (Fig. 3.10d) at δ 56.62/ 56.58 ppm. The lignin compounds, particularly those linked to arabinosyl side chains through ferulic acid bridges, were reflected by carbon signals at δ between 140/160 ppm and 116.6/117.08 ppm. According to Maunu (2002) and Sun et al. (2004), the signals associated with ferulic or *p*-coumaric acids groups and $-\text{CH}_3-$ in $\text{Ar}-\text{COCH}_3$ were assigned to δ 26-49 ppm and δ 115.38 and δ 17.55/17.69 ppm in the ^{13}C NMR spectra of xylan extracted from bagasse by the Lopez method (Fig. 3.9b (i)),

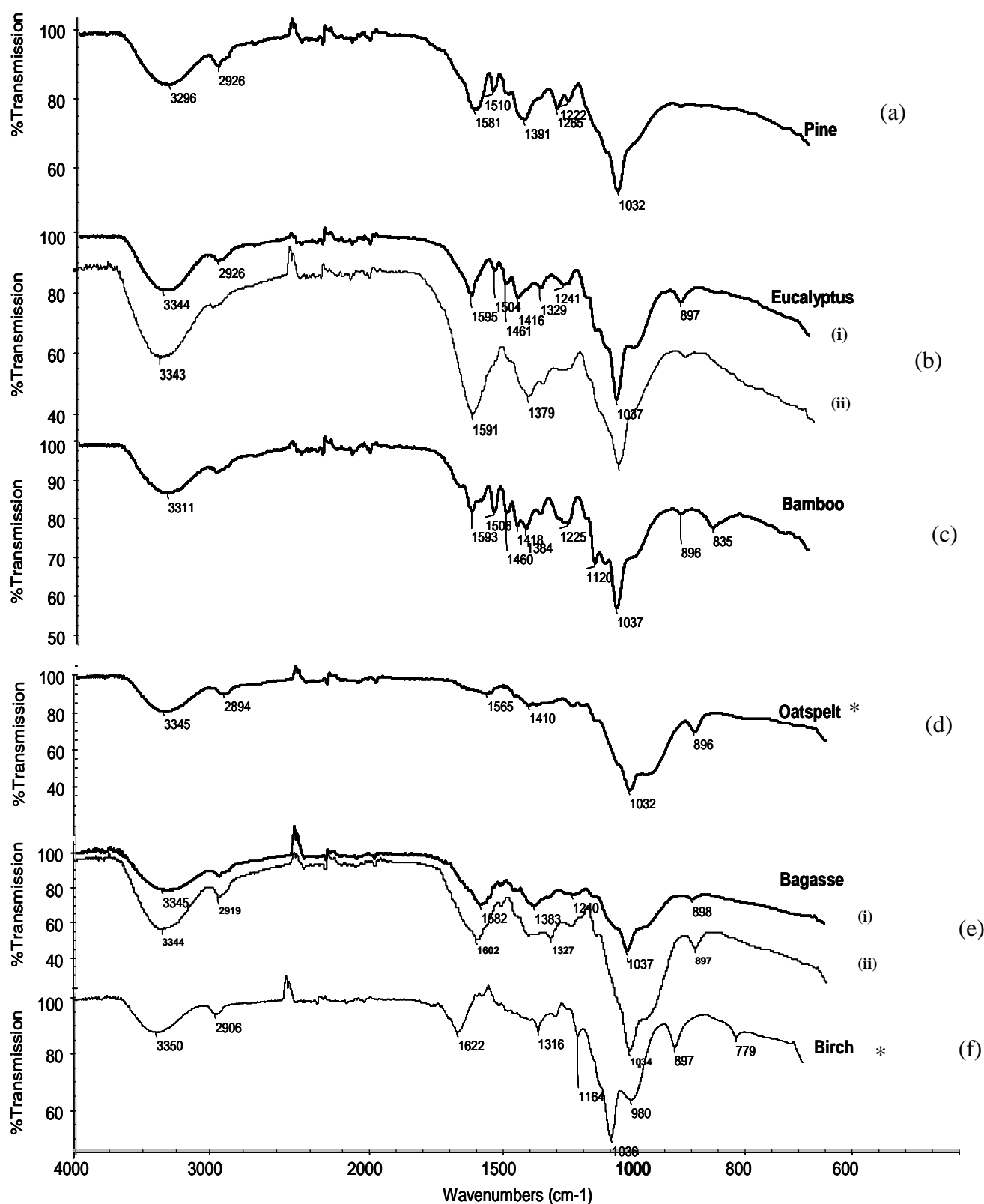


Figure 3.11: FTIR spectra of xylan extracted from different types of lignocellulosic materials (from top) (a) *P. patula* (Pine), (b) *E. grandis* by Hoiije method (i) and *E. grandis* by Lopez method (ii), (c) bamboo, (d) oat spelt xylan*, (e) bagasse by Hoiije method (i) and bagasse by Lopez method (ii) and (f) birch xylan*. The spectra were interpreted according to Fengel and Wegener (1989), Sun et al. (2004^{ab}), Xu et al. (2000), and Sims and Newman (2006). * denotes reference xylan.

xylan fractions extracted from *E. grandis* (Fig. 3.9d), bamboo (Fig. 3.10b) and *P. patula* (Fig. 3.10d). The presence of hexose sugars, such as galactose and glucose, were identified according to Sun et al. (2004^{ab}) in the ¹³C NMR spectra of xylans extracted from *E. grandis* by the Hoiye method (Fig. 3.9d (ii)) and by the Lopez method (Fig. 3.9d (i)) at δ between 69 and 71 ppm. The FTIR spectra of the xylan fractions extracted from *P. patula* (Fig. 3.11a), *E. grandis* (Fig. 3.11b), bamboo (Fig. 3.11c) and bagasse (Figs. 3.11d), assigned according to Fengel and Wegener (1989), Xu et al. (2000), Sun et al. (2004^{ab}) and Sims and Newman (2006), displayed characteristic bands for xylan residues such as those associated with β -glycosidic linkages reflected at $\approx 897\text{ cm}^{-1}$ (except for FTIR spectra of xylan extracted from *P. Patula*). The spectra of xylans extracted from *P. patula* (Fig. 3.11a), *E. grandis* (Figs. 3.11b), bamboo (Fig. 3.11c) and bagasse (Figs. 3.11d) displayed signals in the band region between $1600\text{ and }1200\text{ cm}^{-1}$, which according to Fengel and Wegener (1989), Xu et al. (2000) and Sun et al. (2004^{ab}) is a region associated with aromatic compounds that originate from lignin fractions. Signals arising from syringyl ring breathing with $\text{C}_{\text{Ar}}\text{-OCH}_3$ and methoxyl groups in lignin were reflected at band wavelengths of 1329 cm^{-1} and $1591\text{-}1595$ and $1460\text{-}1461\text{ cm}^{-1}$ in the spectra of xylans from *E. grandis* (Fig. 3.11b) and bamboo (Fig. 3.11c). The bagasse xylan fractions extracted by the Hoiye method (Fig. 3.11e (i)) and Lopez method (Fig. 3.11e (ii)) showed signals of varying intensities in the $1600 - 1200\text{ cm}^{-1}$ wavelength region. In addition, the spectra for xylan extracted from bagasse by the Lopez method (Fig. 3.11e (ii)) reflected a relatively strong intensity band for C-H stretching vibrations at 2919 cm^{-1} . The FTIR spectra for xylan extracted from *E. grandis* by the Lopez method [Fig. 3.11b (i)] displayed two intense signals associated with lignin compounds at 1591 and 1379 cm^{-1} while in the spectra for xylan extracted from *E. grandis* by the Hoiye method (Fig. 3.11b (ii)) had multiple signals of lesser intensity at wavelengths 1595 , 1461 and 1329 cm^{-1} .

3.2.3 Sugar profile of feedstocks pre and post- xylan extraction

The monomeric sugar composition of *P. Patula*, bagasse, *E. grandis* and bamboo feedstocks pre and post xylan extraction are presented in Figures 3.12a-c. The initial glucose levels of the extractive free *P. patula* (Fig. 3.12a), bagasse (Fig. 3.12b), *E. grandis* (Fig. 3.12c) and bamboo (Fig. 3.12d) were 61.0, 68.0, 59.0 and 66.0% respectively. After extraction of the

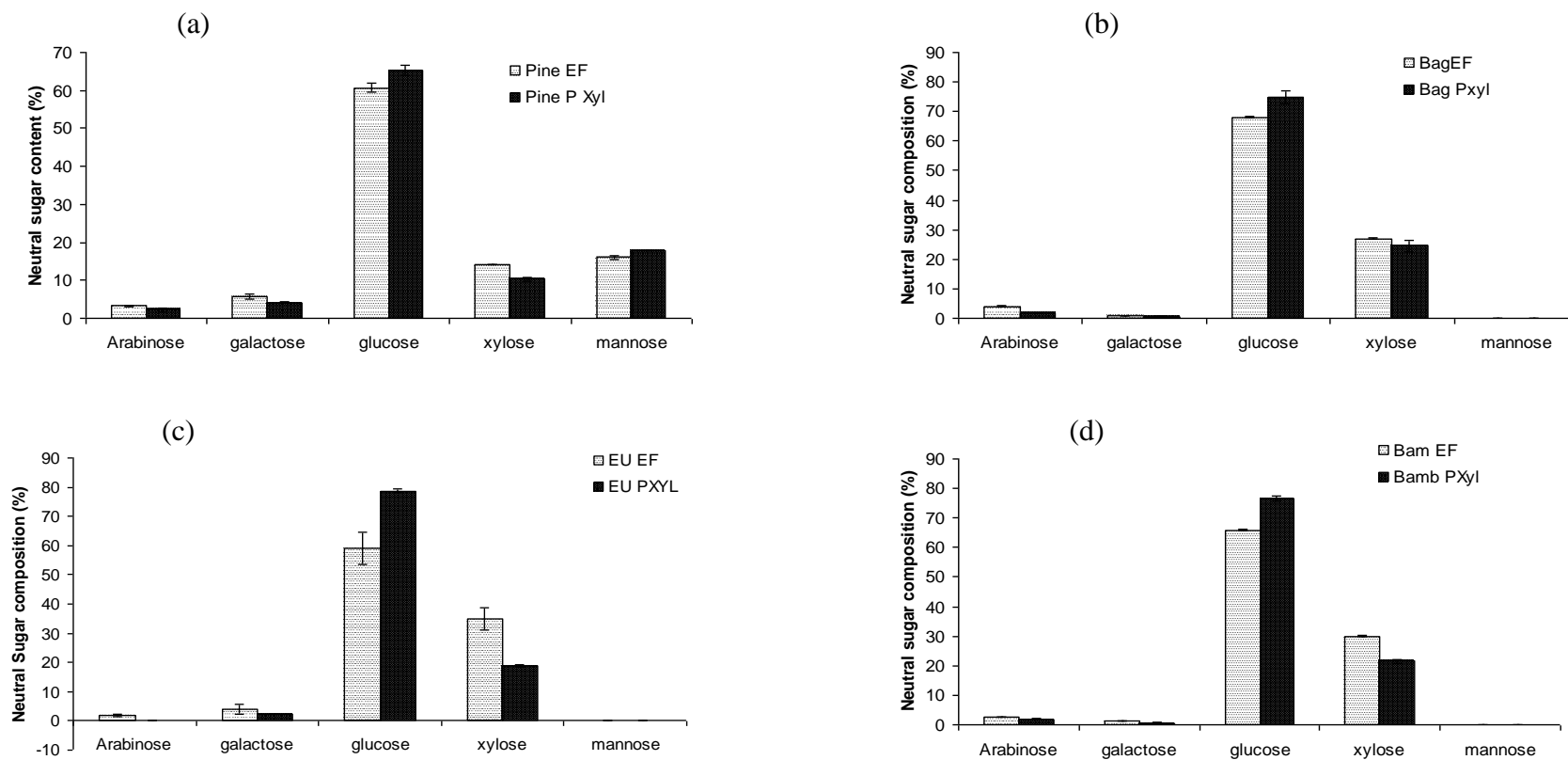


Figure 3.12: Comparison of neutral sugar composition of lignocellulosic materials before (EF) and after xylan extraction (Pxyl) for (a) *P. patula* (Pine), (b) bagasse (Bag), (c) *E. grandis* (EU) and (d) bamboo (BM). Quantification of monomeric sugars was determined from extractive free feedstocks.

xylan using the Hoiye method, the glucose levels of the feedstock cellulolignin residues were 65.0, 75.0, 79.0 and 76.0% respectively. The xylose composition of the cellulolignin residue decreased from 14.0 to 10.0% in *P. patula* (Fig. 3.13a), from 27.0 to 25% in bagasse (Fig. 3.3b), from 35 to 19% in *E. grandis* (Fig. 3.3c) and from 30.0 to 22.0 in bamboo (Fig. 3.3d). Furthermore, low levels of arabinose and galactose were observed in the cellulolignin residue compared to the levels in the initial feedstock material (Figs. 3.12a-d). The presence of mannose was detected in *P. patula* feedstock, the proportion of which increased after xylan extraction 16.0% to 18% (Fig. 3.12a). Uronic acid sugars were detected in all the four lignocellulosic feedstock materials (Table 3.1). The highest and lowest content of uronic acids (10 and 6% respectively) were found in *E. grandis* and bagasse feedstocks respectively (Table 3.1).

3.2.4 Structural composition of feedstocks pre and post xylan extraction

The solid state ^{13}C -CP/MAS NMR spectra for unprocessed, extractive free and xylan extracted residues (cellulolignin) of *P. patula* (Fig. 3.13a), bagasse (Fig. 3.13b), *E. grandis* (Fig. 3.13c) and bamboo (Fig. 3.13d) feedstocks displayed comparable high intensity characteristic signals for the six carbon resonances of the anhydrous glucose ring in cellulose (Figs. 3.13a-d). The signals for the carbon resonances were assigned according to Larsson et al. (1999) and Atalla and Isogai (2005) beginning from the upfield of resonance for C-6 at chemical shift (δ) 60-70 ppm, a cluster of C-2, C-3 and C-5 of the ring carbons other than those anchoring the glycosidic linkage at δ 70-81 ppm, C-4 resonances at δ 81-93 ppm and the anomeric carbon C-1 at δ 102-108 ppm (Figs. 3.13a-d). The C-6 resonances doublets representing less ordered (amorphous) cellulose (upper field) and the ordered (crystalline) cellulose (downfield) (Atalla and Isogai, 2005) were better resolved in the spectra of *P. patula* (Fig. 3.13a) than in the spectra of bagasse (Fig. 3.13b), *E. grandis* (Fig. 3.13c) and bamboo (Fig. 3.13d). Furthermore, characteristic signals for acetyl groups, aliphatic groups, methyl (CH_3) arising from lignin residues, C-1 of arabinose residues, aromatic compounds from lignin residues and C-6 of uronic acid residues or carbonyl groups from xylan were identified in the spectra of the unprocessed raw materials at δ 20-22 ppm, δ 30-40 ppm, δ 50-60 ppm, δ 110 -120 ppm, δ 140-160 ppm and δ 170-190 ppm respectively (Figs. 3.13a-d) (Liitia et al., 2001; Maunu, 2002; Lahaye et al., 2003; Oliveira et al., 2008).

The line and splitting patterns of carbon resonances for the upfield of C-4 (δ 81-93 ppm) and C6 (60-70 ppm) and of the acetyl groups (δ 20-22 ppm) in the ^{13}C -CP/MAS NMR spectra for unprocessed feedstocks, changed with the removal of extractives (Figs. 3.13a-d). The signals for acetyl, aliphatic, methyl, aromatic, C-6 of uronic/carbonyl groups at δ 20-22, 30-40, 50-60, 140-160 and 170-190 ppm respectively in the ^{13}C -CP/MAS NMR spectra of the feedstocks either disappeared or reduced in intensity after xylan extraction (Figs. 3.13a-d). However, in ^{13}C -CP/MAS NMR spectra of bagasse, the signals for carbon resonances of aliphatic groups (δ 30-40 ppm) sharpened and of methyl groups (δ 40-50 ppm) completely disappeared after xylan extraction (Fig. 3.13b). Whereas, the signal for methyl group reduced in intensity in ^{13}C -CP/MAS NMR spectra of *P. patula*, *E. grandis* (Fig. 3.13c) and bamboo (Fig. 3.13d) (Figs. 3.13a, c and d).

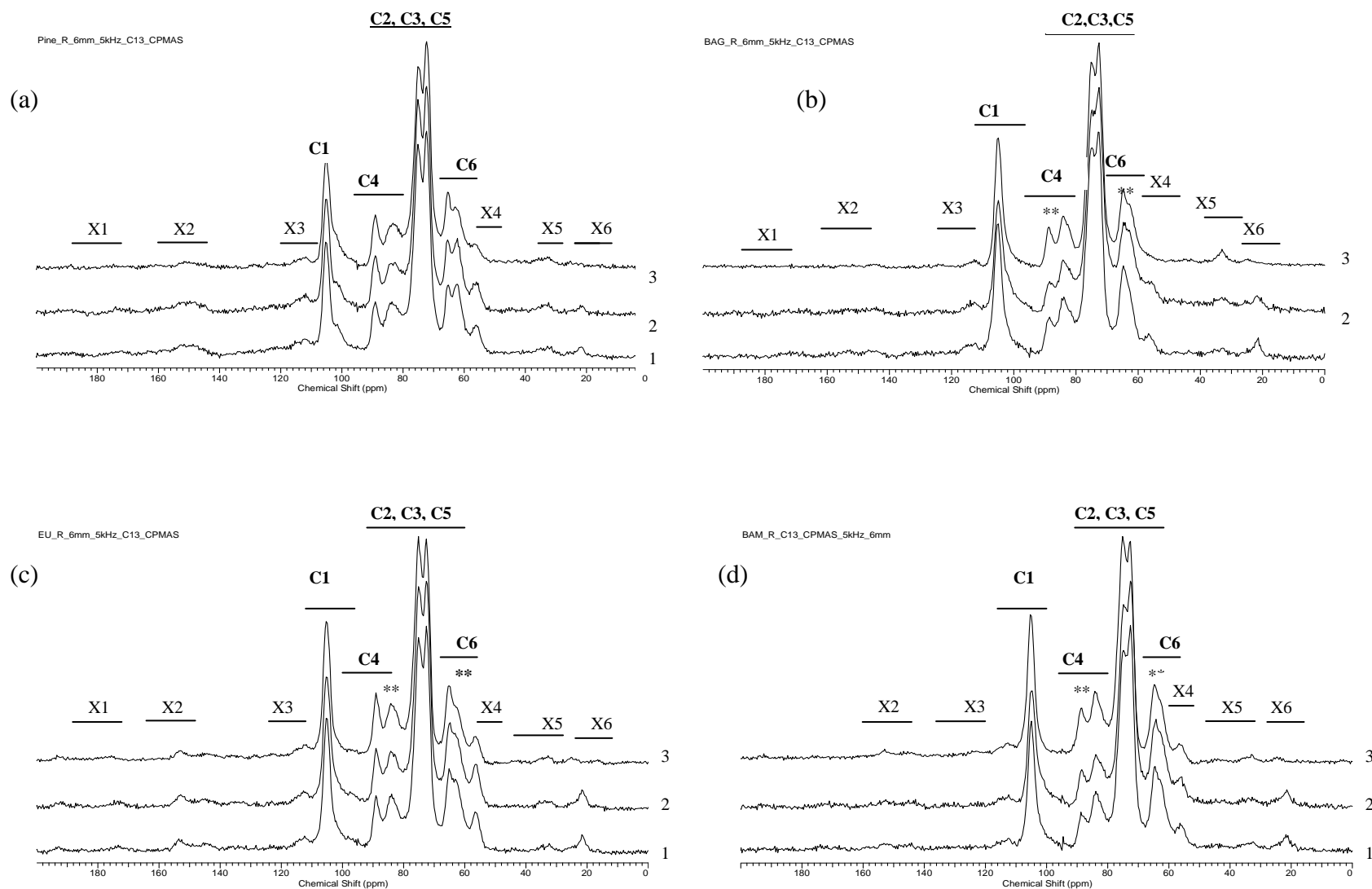


Figure 3.13: Solid state ^{13}C -CPMAS NMR spectra showing the effect of mild alkali xylan extraction by Hoiye method on the integrity of cellulosic fibres in (a) *P. patula*, (b) Bagasse, (c) *E. grandis* and (d) bamboo. The spectra 1, 2, and 3 denote: raw material, extractive free material, and post xylan extracted material and ** denotes upfield peaks for resonances of carbon in glucose units of less ordered cellulose. The assignment of the spectra was based on Larsson et al. (1999), Atalla and Isogai (2005), Liitia et al. (2001), Maunu (2002), Lahaye et al. (2003), and Oliveira et al. (2008). Signals X1 = C6 of MeGlcA residue, X2 = lignin aromatic groups, X3 = arabinose, X4 = Methyl group from MeGlcA or lignin, X5 = aliphatic groups, X6 = acetyl group.

3.3 DISCUSSION

3.3.1 Xylan extraction efficiency

The structural properties and chemical composition of *Eucalyptus grandis*, *Pinus patula*, *Bambusa balcooa* and bagasse found in South Africa were elucidated for extraction of xylans. The chemical composition of the feedstocks showed that bagasse, *E. grandis* and bamboo contained almost twice as much xylan (pentosan) as *P. patula* (Fig. 3.2c). The xylan content, in particular of the *E. grandis*, was higher accounting approximately 20% of biomass (dry weight) (Fig. 3.2c) compared to the xylan content of $\leq 15\%$ of *E. grandis* from Brazil (Emmel et al., 2003), Chile (Ferraz et al., 2000) and China (Yu et al., 2010). The high xylan content renders the South African feedstock reliable sources for extraction of speciality xylans. The production of xylans from these feedstocks could diversify the spectrum of the lignocellulosic products produced in the South African pulp and paper and timber industries.

The Højje and Lopez mild alkali-low temperature methods originally developed for extraction of arabinoxylans from barley straw and barley husks (De Lopez et al., 1996; Højje et al., 2005) were adaptable for extraction of the water soluble polymeric xylans from wood and grass lignocellulosic feedstocks found in South Africa. The Højje method extracted water soluble arabinoglucuronoxylans from bagasse, bamboo and *P. patula* feedstocks and water soluble *O*-acetyl-4-*O*-methyl glucuronoxylan from *E. grandis*. Similarly, the Lopez method was capable of extracting water soluble arabinoglucuronoxylans from bagasse and water soluble *O*-acetyl-4-*O*-methyl glucuronoxylan from *E. grandis* (Fig. 3.2d). Therefore, both the Højje and Lopez xylan extraction methods could be optimised for extraction of xylans from a wide range of feedstocks. However, the Højje method extracted the xylans from the South African feedstock with the highest efficiency compared to the Lopez method. The results showed that efficiencies of extracting the xylans from bagasse and *E. grandis* feedstocks by the Højje method were 2.3 times the efficiency obtained by isolating the xylans using the Lopez method (Fig. 3.2d). The higher xylan extraction efficiency for isolation of xylans achieved by the Højje method compared to the Lopez method was possibly because of the inclusion of the HCl pre-treatment step for the removal of waxes and fats and the NaClO₂ delignification step for removal of lignin prior to the

solubilisation of the xylan with NaOH containing 0.5% sodium borohydride (NaBH_4) (Fig. 3.1a). The removal of extractives and the delignification of the feedstocks prior to xylan solubilisation reduce resistance of the xylan polymers to attack by isolation chemicals. Consequently, xylan is extracted in high yields with reduced contamination from degraded compounds of lignin and extractives. The effects of extractives and lignin to xylan extraction such as low yields and purity have been highlighted before by Browning (1967), Du Toit and Olivier (1984), Talahash and Koshijima (1988) Lawoko (2005) and Balakshin et al., (2008). Therefore, the combined delignification and xylan solubilisation step of the Lopez method, (Fig.3.1b) might have been the xylan yield and quality limiting factor.

The Hoijs and Lopez xylan extraction methods were both more effective in extracting arabinoglucuronoxylans from the feedstocks compared to glucuronoxylans (Fig. 3.2d). The extractability of xylans is restricted by their physical structure and inter-linkages with other cell-wall components (Ebringerová et al., 2005). The results showed that the extraction efficiencies of the xylans extracted by both the Hoijs and Lopez methods from bagasse were twice as much as that of *E. grandis* (Fig. 3.2d). The higher extractability of arabinoglucuronoxylans from bagasse than that of glucuronoxylan from *E. grandis* rather contradicts with the explanation given by Timell (1967) who indicated that hardwood xylans (glucuronoxylans) are easily extracted with alkali solvents in pure state and in higher yield compared to arabinoglucuronoxylans from softwood and some grasses because of reduced side chain substitution. In particular, the difficulty to extract xylans from lignocellulosic materials in higher yield and purity is often associated with complex xylan substitution pattern and high lignin content, which result into formation of lignin-carbohydrate complexes (LCC) that resist extraction in most extraction solvents (Timell, 1967; Joseleau et al., 1994; Ebringerová et al., 1998). The results in Table 3.1 showed that the xylans extracted from the bagasse, *P. patula* and bamboo feedstocks were highly substituted compared to the xylan extracted from the *E. grandis* (Figs. 3.9 & 3.10). Therefore, the higher xylan yield observed, for instance, with *P. patula* compared to the *E. grandis* feedstocks could probably be associated with the presence of high molecular mass acid insoluble fraction estimated in (Fig. 3.3).

However, the relatively lower extraction efficiency for arabinoglucuronoxylans extracted from bamboo estimated at 20% compared to 71% of *P. patula* and 65% of bagasse could be attributed to the unique complex substitution pattern of the arabinoglucuronoxylans of bamboo compared to arabinoglucuronoxylans derived from softwoods and other grasses (Wilkie, 1979; Salmela et al., 2008). The bamboo xylan is known to be rich in syringyl lignin and contains both the single and oligomeric side chains consisting of arabinose, galactose, xylose, MeGlcA and *p*-coumaric acid (Wilkie and Woo, 1977; Wilkie, 1979, Fengel and Shao, 1985). Therefore, the arabinoglucuronoxylan extracted from bamboo are prone to develop more complex crosslinkages with the lignin in the cell wall matrix compared to arabinoglucuronoxylans from other sources (Wilkie, 1979). Consequently, breaking of such linkages would require use of high alkaline charge (Fengel and Wegener, 1989) at temperatures as high as 150°C (Aoyama and Seki, 1999; Vu et al., 2004). Such conditions could not be met under the operating conditions of the Hoiye method.

The efficiencies of extracting xylans from *P. patula* and *E. grandis* were higher compared to efficiencies of extracting xylans from related wood feedstocks. In this study, the xylans were extracted from *P. patula* and *E. grandis* feedstocks with extraction efficiencies of 71% and 35% using the Hoiye method (Fig 3.2d), whereas, the efficiencies reported for extracting xylans from other wood sources by application of similar mild alkaline based methods ranged from 23-31.4% (Glasser et al., 2000; Ebringerová and Heinze, 2000). Therefore, at 35 and 71% xylan extraction efficiencies, the recovery of xylan per tonne of *E. grandis* and *P. patula* feedstocks respectively, from mild alkali extraction process would be 70 kg, which is higher than the recovery of xylan of 40-50 kg per tonne reported for aspen chips (Al-Dajani and Tschirner, 2008). However, the extraction efficiency of 65% for extraction of xylan from bagasse of the Hoiye method (Fig. 3.2d) was relatively lower than the xylan extraction efficiency of approximately 90% obtained from extraction of xylan from feedstock using sequential alkaline extraction methods (Sun et al., 2004^a; Peng et al., 2009; Brienza et al., 2009). Therefore, the lower xylan extraction efficiencies for xylans extracted from the South African bagasse feedstocks by the Lopez and the Hoiye methods could in part be attributed to possible incomplete fractionation and recovery of the xylans from the xylan extracts. The recovery of xylan fractions by acidified ethanol precipitation, which in this study was used in the Lopez method, is

considered inefficient because the recovery of the xylan fractions is according to degree of xylan polymerisation and ethanol concentrations (Wilkie, 1979; Peng et al., 2009). Therefore, higher xylan yields could be realised by recovering a wide range of different sized xylan fractions from the xylan extract. Gradient solubilisation and fractionation procedures achieved by varying the alkali and ethanol concentrations have been reported by other workers (Coimbra et al., 1995; Schooneveld-Bergmans et al., 1999; Xu et al., 2006; Peng et al., 2009).

3.3.2 Quality of the extracted xylans

The chemical and structural composition of the extracted arabinoglucuronoxylans from *P. patula*, bagasse and bamboo and glucuronoxylan from the south African *E. grandis* using both the Hoije and Lopez extraction methods, were similar to the chemical and structural composition of xylan fractions extracted from close related feedstocks found in Europe, South America and Asia (Timell, 1967; Wilkie, 1979; Sjöström, 1993; Kuhad et al., 1997; Geng et al., 2006; Ebringerová, 2006). The identities of the extracted xylan fractions, which were defined by the proton and carbon resonances displayed in ^1H NMR and ^{13}C NMR spectra of the extracted xylans showed presence of functional groups such as xylose, methyl, acetyl and arabinose. The signals of the carbon proton resonances of such xylan functional groups displayed in the ^1H NMR and ^{13}C NMR spectra of the extracted xylans, corresponded to those that disappeared or whose intensity reduced in the solid state ^{13}C -CP/MAS NMR spectra of the unprocessed *P. patula* (Fig. 3.13a), bagasse (Fig. 3.13b), *E. grandis* (Fig. 3.13c) and bamboo (Fig. 3.13d) feedstocks after being subjected to the mild alkali-low temperature extraction protocols. In addition, such functional groups corresponded to the sugar composition of the xylans presented in Table 3.1.

However, the presence of the acetyl groups in the xylan extracts from the South African *P. Patula* as displayed in the ^1H NMR and ^{13}C NMR spectra (Figs. 3.10c and d), combined with the presence of the glucose and galactose units (Table 3.1), suggest possible contamination of the extracted xylan with *O*-acetyl galactoglucomanans. The contamination of the xylan by the galactomannans is not uncommon because the *O*-acetyl galactoglucomanans are the main hemicelluloses in softwoods galactoglucomannans, which are known to be less resistant to the alkaline degradation

than xylans as such they are the first to degrade during xylan extraction (Timell, 1967; Fengel and Wegener, 1989; Sjöström, 1993).

The xylans extracted from the feedstocks by the Hoiye method had physical structures that were more suitable for enzyme modification than the xylans extracted by the Lopez method. The xylan fractions extracted with increased brightness are considered more suitable for enzymatic hydrolysis (Brienzo et al., 2009). This is because the colour is considered as one of the indicators reflecting purity. The dark pigmentation of xylan fractions is often associated with the reaction state of the phenolic compounds linked to lignin, extractives and possible presence of hexenuronic acids from alkali hydrolysis of uronic acids in the extracted xylans. The results showed that despite similarities in chemical composition (Table 3.1) and structural composition (e.g. DP >10), the xylans extracted from the bagasse (Fig. 3.4a) and *E. grandis* feedstocks (Fig. 3.4 d) by the Lopez method had darker brown pigmentation compared to the corresponding xylans extracted by the Hoiye method (Figs. 3.4b and 3.4e respectively). Although the xylan extracted from the *P. patula* had brighter pigmentation (Fig. 3.4f) than the xylan extracted from *E. grandis* (Fig. 3.4d), the percentage of the acid insoluble fraction, which is a reflection of the Klason lignin content was higher in the *P. patula* xylan fractions (Fig. 3.3). These results were confirmed with the high intensity signals emanating from lignin fractions displayed in ^1H NMR at δ between 6 and 8 ppm and in ^{13}C NMR spectra at δ between 140-160 ppm for the xylan extracted from the *P. patula* (Figs. 3.10c and d) compared to those of the xylan extracted from *E. grandis* (Figs. 3.9c and d). Therefore, the darker pigmentation of the xylan extracted from *E. grandis* than the one extracted from *P. patula* could be associated with the oxidised state of the lignin rather than the lignin content present in the xylan fractions. Compounds that cause pigmentation of the extracted xylan could be inhibitory to its enzymatic hydrolysis.

Furthermore, side chain removing enzymes work more effectively on water soluble xylan substrates compared to xylan fractions in crystallised state (Tenkanen and Siika-aho, 2000; Ebringerová and Heinze, 2000). The results showed that the xylan extracted from the bagasse (Fig. 3.4a) and *E. grandis* (Fig. 3.4 d) by the Lopez method were in a more crystallized state compared to the xylan extracted by the Hoiye method (Figs. 3.4b and 3.4e). This effect was possibly a consequence of prolonged

processing time, in particular, due to the longer time it took to lyophilise the xylan extracts. The xylan extracts that contain residual ethanol such as those fractionated by the Lopez method have longer lyophilising time because the ethanol has lower freezing point than water. Consequently, strong hydrogen bonds developed with such longer dehydration time leading to the observed crystal-like state as described by Ebringerová and Heinze (2000). Therefore, the darker pigmentation and crystal-like state of the xylan extracted by the Lopez method may not render the xylan extracted from the South African feedstocks suitable for enzyme modification. This is because xylan crystallinity would affect accessibility of the enzymes to the xylan linkages (Tenkanen and Siika-aho, 2000).

3.3.3 Quality of the cellulosic-lignin residue post xylan extraction

The mild alkali-low temperature xylan extraction methods were more selective for the xylan, lignin and extractives than the cellulosic component. The extraction of the xylan by Hoije and Lopez methods altered the chemical composition but without damaging the structural integrity of the cellulosic residues in the feedstock residue. The observation suggests that the cellulosic-lignin residues could be used as raw materials for co-production of xylan with other value added products, thus, befitting the concept of lignocelluloses biorefining (Ragauskas et al., 2006). The results showed that the chemical composition of the cellulosic-lignin (cellulolignin) residues derived from *P. patula* (Fig. 3.12a), bagasse (Fig. 3.12b), *E. grandis* (Fig. 3.12c) and bamboo (Fig. 3.12d) feedstocks after extraction of the xylan by the Hoije method consisted predominantly of glucose units, suggesting that composition of the cellulosic residue was preserved during the xylan extraction. In addition, the solid state ^{13}C -CP/MAS NMR spectra of the unprocessed *P. patula* (Fig. 3.13a), bagasse (Fig. 3.13b), *E. grandis* (Fig. 3.13c) and bamboo (Fig. 3.13d) showed that intensity of the signals for the carbon resonances originating from the glucose units of the cellulosic residues of *P. patula* (Fig. 3.13a), bagasse (Fig. 2.13b), *E. grandis* (Fig. 3.13c) and bamboo (Fig. 3.13d) remained the same after xylan extraction. In contrast,, the signals corresponding to the aromatic and aliphatic compounds that are associated with lignin displayed at δ 30-40, 50-60 and 140-160 ppm, those associated with MeGlcA displayed at δ 177 ppm and those associated with acetyl groups displayed at $\delta \approx 22$ ppm disappeared from the solid state ^{13}C CP/MAS NMR spectra of the original

P. patula (Fig. 3.13a), bagasse (Fig. 3.13b), *E. grandis* (Fig. 3.13c) and bamboo (Fig. 3.13d) feedstocks with the xylan extraction.

Although, the integrity of the main cellulosic structure of the feedstock residue was preserved with extraction of the xylans, it appears the crystallinity and surface properties of the cellulose fibrils were altered. The effect was reflected by the changes in smoothness, splitting pattern and the intensity of the C-4 and C-6 duplets carbon resonances that originated from glucose units at δ 81-90 ppm and 60-70 ppm in the ^{13}C -CP/MAS NMR spectra of the cellulolignin residue of *P. patula* (Fig. 3.4a), bagasse (Fig. 3.4b), *E. grandis* (Fig. 3.4c) and bamboo (Fig. 3.4d). In the ^{13}C -CP/MAS NMR spectra the regions at δ 81-90 ppm and 60-70 ppm displayed carbon resonances for both ordered (crystalline) and less ordered (non-crystalline) cellulose fibrils, which are closely associated with presence of hemicelluloses (Atalla and Isogai, 2005). Therefore, the alterations that occurred to the C-4 and C-6 carbon resonances might suggest alteration of the crystallinity of the cellulose (i.e. the cellulose became more amorphous) due to the xylan extraction. The changes that occurred to the structure of the cellulose residue upon xylan extraction are consistent with the results of Larsson et al. (1999) who found that the removal of xylan during kraft pulping reduced the signal intensities of the carbon resonances in the ^{13}C NMR spectra for both ordered (86-92 ppm) and less ordered (80-86 ppm) cellulose. The reduction in the crystallinity of the cellulose residue and the partial removal of the lignin, acetyl and uronic acids residues associated with the xylan extraction would render the cellulosic residues more susceptible to pulping and bleaching chemicals (Sjöström, 1993). Therefore, the chemical and energy demand for subsequent processing of the cellulolignin residues for pulp and paper making could subsequently be reduced.

3.4 CONCLUSION

The chemical composition and structure of bagasse, bamboo, *E. grandis* and *P. patula* was elucidated. The feedstocks contained extractable xylan of up to 22% of the feedstock on moisture free basis. Arabinoglucuronoxylans were selectively extracted from bagasse, bamboo and *P. patula* whereas 4-*O*-methylglucuronoxylan was extracted from *E. grandis* found in South Africa using the mild alkali–low temperature xylan extraction methods. The xylans was extracted from *E. grandis* and bagasse feedstocks with high efficiency and purity by the Hoiye method compared to the Lopez method, The xylans extracted by both the Hoiye and Lopez methods were polymeric and substituted with arabinose and MeGlcA side chains, the features that could suit enzyme modification. The use of the mild alkali–low temperature xylan was not damaging to the integrity of the cellulolignin residues and, therefore, could enhance co-production of the speciality xylans with other value added cellulosic-lignin based products. Therefore, the ability to extract water soluble polymeric xylans from the bagasse, bamboo, *E. grandis* and *P. patula* feedstocks provides suitable substrates for enzyme modification that would enhance utilisation of xylans in lignocelluloses processes and reduce xylan wastage.

Chapter 4: Production and characterisation of recombinant α -L-arabinofuranosidase with xylan polymeric substrate specificity

ABSTRACT

An *Aspergillus niger* D15 [*abfB*] strain was constructed to express the *abfB* gene under the transcriptional control of the glyceraldehyde-3-phosphate dehydrogenase promoter (*gpd_P*) and glucoamylase terminator (*glaA_T*). The secretion of the recombinant AbfB was growth associated, which increased with enrichment of concentrated corn steep liquor in the 2x minimal medium. During the shake flask and 10 L bioreactor fermentation cultures, the *A. niger* transformants produced recombinant α -L-arabinofuranosidase (AbfB) with volumetric activities of up to 10.0 and 8.0 nkat mL⁻¹ respectively, against *p*-nitrophenol arabinofuranoside (*p*NPA). The biomass specific activity was up to 366 nkat g⁻¹ (DW). The crude recombinant AbfB protein concentration ranged between 0.12-0.45 g L⁻¹ depending on the cultivation media. The specific activity of the crude recombinant AbfB was 2.9 U mg⁻¹_{protein}. The recombinant AbfB was present as a single pure species on 10% SDS-PAGE when using Coomassie staining, with an estimated molecular mass of 67 kDa. The recombinant AbfB displayed optimal activity at 40-55°C and pH 3.0-pH 5.0 and was stable at 60°C and pH 6.0. The recombinant AbfB displayed broad polymeric xylan substrate specificity releasing 20% of the available arabinose from low viscosity wheat arabinoxylan and oat spelt arabinoxylan, 9% from bagasse arabinoglucuronoxylan, 7% from larchwood arabinogalactan and debranched arabinan and 5% from bamboo and *P. patula* arabinoglucuronoxylans. More than 95% of the recombinant AbfB activity against *p*NPA was recyclable after xylan hydrolysis at 40°C for 16 h. Therefore, the recombinant *A. niger* D15 [*abfB*] strain has presented a system for secretion of the AbfB with improved purity under the transcriptional control of the *gpd_P* promoter and *glaA_T* terminator. Such recombinant AbfB enzyme has application for selective removal of arabinose side chains that would allow customised modification of functional properties of polymeric xylans from various feedstocks.

4.0 INTRODUCTION

The α -L-Arabinofuranosidase (EC 3.2.1 55) is an important accessory enzyme, alongside α -D-glucuronidase and acetyl esterases, for synergistic action with xylanases during complete xylan hydrolysis. The α -L-arabinofuranosidase is responsible for cleavage of arabinose side chains from the xylan backbone chain (Tagawa and Kaji, 1988). Most of the known α -L-arabinofuranosidases remove arabinose groups only from oligomeric xylan residues, such that hydrolysis of polymeric xylans is often performed in cooperation with the other xylan main chain depolymerising enzymes such as endo- β -1,4-xylanases (β -xylanases; EC 3.2.1.8) and β -xylosidases (EC 3.2.1.37) (Gilead and Shoham, 1995; De Vries and Visser, 2001). Consequently, the application of such α -arabinofuranosidases is limited to facilitating complete hydrolysis of the oligomeric xylans generated by β -xylanases, to xylose units in bio-bleaching of pulp fibres that enhances the removal of residual lignin (Puls et al., 1990; Haltrich et al., 1996), clarification of juices in juice making (Saha, 2000), enhancement of digestibility of feedstock in animals (Brice and Morrison, 1982; Gilead and Shoham, 1995) and release of aroma enhancing compounds in wine making (Birgisson et al., 2004). In addition, the application of the α -L-arabinofuranosidase was done to facilitate production of xylose monomeric sugars from the xylans for use in xylitol and biofuels production (Howard et al., 2003). However, the α -L-arabinofuranosidases that selectively remove arabinose groups from xylans are now required to diversify the product spectrum of xylans in lignocelluloses processing in order to match those of cellulose and starch. In particular, the xylans are needed for production of insoluble xylan hydrogels for use as biodegradable coatings and encapsulation matrices in industrial applications in view of increased environmental concerns associated with fossil fuel derived polymers.

The presence and distribution of the arabinose side chains in the main xylan chain is one of the limiting factors for the lack of large scale industrial applications of the xylans as coatings and encapsulation matrices compared to cellulose and starch. Currently, the removal of arabinose side chains from polymeric xylans is achieved through chemical methods, which due to non-selectivity of their reactions offer limited control over the quantity and quality of the produced precipitated xylan hydrogels. Therefore, α -L-arabinofuranosidases with the ability to remove the

arabinose side chains from polymeric xylan in the absence of the main chain degrading enzymes are of technological importance for targeted reduction of the solubility of xylans.

The α -L-Arabinofuranosidases with polymeric xylan substrate specificity belong to glycoside hydrolases (GH) family 54 (Biely, 2003). The α -L-arabinofuranosidases (AbfBs) of family GH 54 have shown the ability to cleave off arabinose side chains that are α -1,2-linked or α -1,3 linked to xylose units at the non-reducing terminal end of the xylan chain from both oligomeric and polymeric xylans, α -1,3 linked arabinose side chains to arabinogalactans and α -1,5 linked arabinose side chains in L-arabinan (Luonteri et al., 1995; Margolles-Clarke et al., 1996; Kaneko et al., 1998; Biely, 2003). Such AbfBs are known for their broad substrate specificity as such could be used for selective hydrolysis of arabinose substituted xylans extracted from a wide range of lignocellulosic materials. Despite having broad substrate specificity, the AbfBs are selective of the linkages and the position of the arabinose side groups they attack as such could offer xylan modifying technology with a higher degree of control over the quality and quantity of the xylan products

The large scale application of the AbfBs would require large quantities of their extracellular production and free of endo- β -xylanase and β -xylosidase contamination. Such selective AbfB production can be achieved in recombinant microbial enzyme production systems. Since the *abfB* gene for expression of the AbfBs was first described (Tagawa and Kaji, 1988; Flipphi et al., 1993), it has been cloned in various microbial production systems but with limited success (Manin et al., 1994; Crous et al., 1996; Gouka et al., 1996; Van Den Hombergh et al., 1997; Wang et al., 2005). *Aspergillus niger* microbial systems have been widely used in many industrial applications to improve production levels of industrial enzymes due to the ability to produce extracellular functional homologous and heterologous proteins in sufficient quantities (Wong and Saddler, 1993; Verdoes et al., 1995; Punt et al., 2002). Furthermore, the successful use of inexpensive medium for their growth makes *A. niger* one of the most cost effective microbial systems for large scale industrial enzyme production (van den Hombergh et al., 1997). In addition, *A. niger* has GRAS (Generally Regarded as Safe) status and FDA (Food and Drug Administration) approval, which renders its eligibility for the international market. However, wild type

strains of *A. niger* inherently secrete a wide variety of extracellular enzymes, which include β -xylanases and proteases (Verdoes et al., 1995; Margolles–Clark et al., 1997; Punt et al., 2002). The extracellular β -xylanases are the main chain degrading enzymes that could compromise the selective hydrolysis of the polymeric xylans, whereas, the extracellular protease are protein degrading enzymes that could reduce the specific activity of other enzymes (Verdoes et al., 1995). Consequently, extensive and costly downstream purification steps are necessary when working with the native *A. niger* as a host. Such processes increase the cost of enzyme production (Hatti-Kaul and Mattiasson, 2001).

The purpose of this study was to develop a recombinant microbial system for extracellular production of the AbfB enzyme that is free of xylan main chain degrading enzymes and therefore, can be used for custom modification of the functional properties of xylans extracted from lignocellulosic feedstocks found in South Africa. In the study, the protease-deficient, non-acidifying pH mutant *A. niger* D15 (Wiebe et al., 2001) was genetically transformed to be the host for the extracellular overproduction of the AbfB of *A. niger*. The strain *A. niger* D15 has previously been used successfully for the production of both heterologous and homologous enzymes (Rose and Van Zyl, 2002; Rose and Van Zyl, 2008; Alriksson et al., 2009; Van Zyl et al., 2009). The *abfB* gene was cloned and expressed under the transcriptional control of glyceraldehyde-3-phosphate dehydrogenase promoter (*gpd_P*) of *A. nidulans* and glucoamylase terminator (*glaA_T*) of *Aspergillus awamori*. The growth characteristics of the *A. niger* D15[*abfB*] strain and the AbfB production levels were studied in shake flasks and 10 L bioreactor on defined standard media and corn steep liquor enriched medium. Furthermore, the recombinant AbfB's production characteristics, substrate specificity and dependency, optimal operating pH and temperature and stability and recyclability in storage and during hydrolysis were assessed.

4.1 MATERIALS AND METHODS

4.1.1 Strains and media compositions

The genotypes of the bacterial and fungal strains as well as the plasmids used in this study are summarised in Table 4.1. Recombinant plasmids were constructed and amplified in *Escherichia coli* DH5 α . The *E. coli* was cultivated at 37°C in LB medium (1% yeast extract, 1% tryptone and 0.5% NaCl) on a rotary shaker at 100 rpm, supplemented with 100 $\mu\text{g L}^{-1}$ ampicillin. The *A. niger* strains were maintained at 30°C in minimal media (MM) and on spore plates (Rose and Van Zyl 2002). Transformants were obtained as described in Rose and Van Zyl (2002) and selected on MM lacking casamino acids and uridine. Cultivation took place in Erlenmeyer shake flasks (125 mL) containing 30 mL of double strength traditional minimal medium (2xMM) supplemented with 10% glucose and an inoculum of 1×10^6 spores mL^{-1} was added. The cultivation of the *A. niger* strains took place at 30°C on a rotary shaker (120 rpm).

Table 4.1: The genotype and sources of the strains and plasmids used in transforming *A. niger* D15 strain.

Strain:	Genotype	Reference
<i>A. niger</i> D15	<i>pyrG prtT phmA</i> (nonacidifying)	Wiebe et al. (2001) ATCC 9029
<i>A. niger</i> van Tieghem	Wild-type	ATCC 10864
<i>A. niger</i> D15[pGTP]*	<i>A. niger</i> D15 with <i>gpdP-glaAT</i>	Rose and Van Zyl (2008)
<i>A. niger</i> D15[abfB]*	<i>A. niger</i> D15 with <i>gpdP-abfB-glaAT</i>	This study
<i>E. coli</i> DH5 α	<i>supE44 ΔlacU169 (0/80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Sambrook et al. (1989)
Plasmids:		
pBS-pyrGamdS	<i>bla pyrG amdS</i>	Plüddemann and Van Zyl (2003)
pGTP	<i>bla gpdP-glaAT pyrG</i>	Rose and Van Zyl (2008)
pGTP-abfB	<i>bla gpdP-abfB-glaAT pyrG</i>	This study

*plasmids were integrated

Spore production of the *A. niger* D15 strains was first done on plates (Fig. 4.1) . The spores were later inoculated on rice sporulation medium (20 g of rice containing 8 mL

of 0.1% (w/v) urea). The rice was autoclaved at 121°C for 15 min before inoculation. Spores were harvested (in saline solution) after incubation at 30°C for a period of 3-6 days and stored at 4°C. *A. niger* spores were inoculated in 2xMM medium enriched with concentrated corn steep liquor (CCSL) (African Products, South Africa), which was prepared according to a modified version of Gurlal et al. (2006) protocol.



Figure 4.1: Sporulation plates containing minimal medium (MM) with nitrate for production of *A. niger* spores. The plates were incubated at 30°C until 80% of the surface was covered with spore growth.

The CCSL was autoclaved and filtered (0.22 µm pore size) prior to addition to the minimal medium at 1%, 2% and 10% (w/v). Culture samples were taken at 24 h intervals for 7 days and the AbfB activity determined. Unless otherwise stated, the CCSL optimised medium (2% w/v CCSL) was used in the subsequent cultivations of *A. niger*.

4.1.2 Plasmid construction

Standard protocols were followed for all DNA manipulations (Sambrook et al., 1989). The construction of the plasmids and the fungal strains was similar to that described in detail in Rose and van Zyl (2002). Briefly, the *pyrG* gene had been retrieved from pBS-pyrGamdS (Plüddemann and Van Zyl, 2003) via PCR and was cloned into the *EcoRI* site on plasmid pGT (Rose and Van Zyl, 2002), generating pGTP. The *abfB* gene was amplified from the genome of *A. niger* ATCC10864 using PCR and cloned into the *NotI* site of pGTP, generating pGTP-AbfB. The transcriptional control of *abfB* is directed by the glyceraldehyde-3-phosphate dehydrogenase promoter (*gpd_P*) of *A. nidulans* and glucoamylase terminator (*glaA_T*) of *A. awamori* (Fig. 4.2). To

select the highest producer, a total of 100 *A. niger* D15 [abfB] transformants were screened for extracellular AbfB activity.

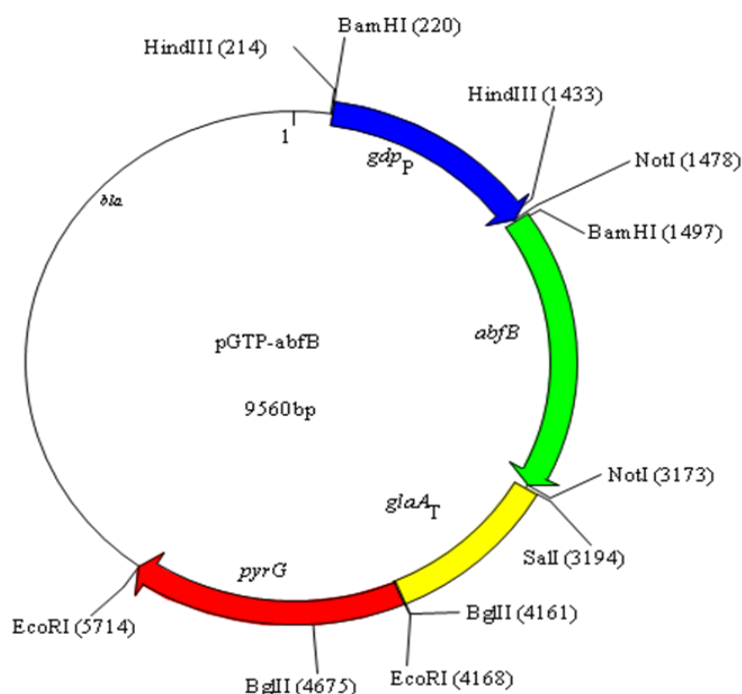


Figure 4.2: Schematic representation of plasmid pGTP-AbfB showing *abfB* cloning site. The *abfB* gene was cloned into the *NotI* site of pGTP, with the transcriptional control of *abfB* directed by the glyceraldehyde-3-phosphate dehydrogenase promoter (*gdp_P*) of *A. nidulans* and glucoamylase terminator (*glaA_T*) of *A. awamori*. The pGTP-AbfB vector was integrated into the genome of *A. niger* D15 in multiple sites.

4.1.3 α -L-Arabinofuranosidase activity

The AbfB activity was determined in a colorimetric assay by measuring the release of *p*-nitrophenol (Sigma) from *p*-nitrophenyl- α -arabinofuranoside (*p*NPA, Sigma). The 100 μ L reaction mixture, consisted of 25 μ L 5 mM *p*NPA (in 0.05 M citrate buffer, pH 5.0), 25 μ L deionised water (dH₂O), 25 μ L of appropriately diluted supernatant and 25 μ L 0.05 M citrate buffer (pH 5.0) except for AbfB activity determined during medium optimization where 0.05 M citrate buffer pH 6.0 was used. The reaction was performed at 40°C for 10 min and was terminated by the addition of 100 μ L saturated sodium tetraborate (Sigma). The mixture was diluted five times and the absorbance measured at 405 nm wavelength using a spectrophotometer. The AbfB activity was determined from a standard curve using *p*-nitrophenol as standard under similar assay conditions. The AbfB activity was calculated as the amount of nmole *p*NP released

from ρ NPA per mL per second (nkat mL^{-1} , conversion factor: 1 Unit = 16.67 nkat). Commercial α -L-arabinofuranosidase from *A. niger* (E-AFASE, Megazyme) was used as a positive control.

4.1.4 AbfB characterisation

Total protein in enzyme preparations was determined according to Bradford (1976) using Biorad protein assay with bovine serum albumin (BSA) as the standard protein. Enzyme preparations were subjected to a 10% SDS-PAGE separation prepared according to Laemmli (1970). The protein species were initially stained with Coomassie Brilliant Blue R250 and subsequently with silver stain (Bio-Rad Silver Stain kit). The molecular weight (M_w) was estimated from the gel using BenchMarkTM prestained protein marker (Invitrogen and Prestained protein ladder, Inqaba). Theoretical M_w and isoelectric point (pI) of the AbfB was estimated from the original protein sequence (protein length of 499 bases) using DNAMAN sequence analysis computer software. The difference between the theoretical M_w and the estimated SDS-PAGE M_w gave an indication of the extent of glycosylation of the protein.

The recombinant AbfB was characterised with respect to optimal pH (McIlvaine, 1921) and temperature and pH and temperature stability under standard assay conditions for ρ NPA. The effect of substrate concentration on AbfB activity was determined by preparing ρ NPA at varying concentrations in 0.05 M citrate buffer (pH 5.0). The kinetic properties such as maximum activity (V_{\max}) and Michaelis-Menten constant (K_m) of partially purified and crude enzyme were determined from the plot of release of ρ NP as a function of ρ NPA substrate concentration.

4.1.5 β -Xylanase activity and total sugar determination

The amount of β -xylanase activity present in the AbfB preparations was determined by measuring the release of reducing sugars from birchwood xylan (Roth, Germany) using dinitrosalicylic acid (DNS) [Miller, 1959; Bailey et al. 1992] and xylose (Merck) was used as standard. β -Xylanase activity was calculated as amount of nmoles of xylose released per mL per second ($\text{nmoles mL}^{-1}\text{sec}^{-1}$) presented as nkat mL^{-1} . Residual sugar concentration in the fermentation cultures was determined by the phenol-sulphuric acid assay (Dubois et al., 1956).

4.1.6 Production of AbfB in pellet and mycelial morphology

Pellet biomass formation (Fig. 4.3a) was achieved by inoculating the medium with 1×10^6 spores mL^{-1} and incubating the shake flasks at 30°C on a shaker (120 rpm).

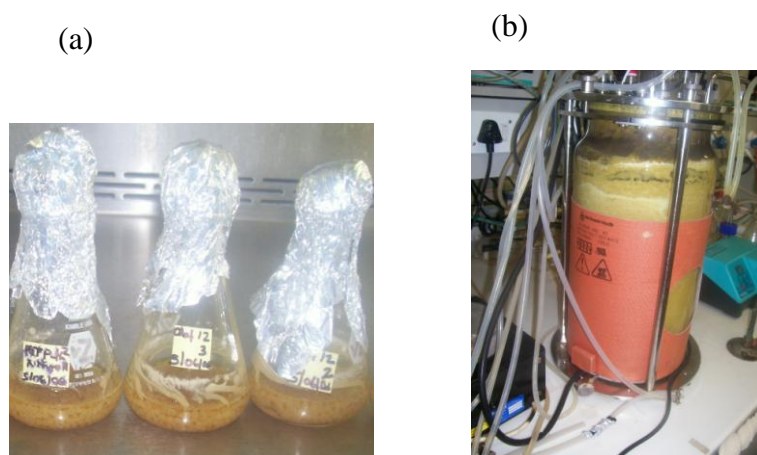


Figure 4.3: *Aspergillus niger* cultivation for extracellular production of recombinant α -L arabinofuranosidase (a) pellet and (b) mycelia morphologies.

The cultivation medium had an initial pH of 5.5-6.0 and 5.0 for 2xMM and CCSL enriched respectively. Mycelial production (Fig. 4.3b) of AbfB by *A. niger* D15[abfB] was done in a 14-L capacity stirred tank bioreactor (BIOFLO 110, New Brunswick Scientific company, Inc, USA) with 10 L working volume containing 8 L of optimised 2xMM medium enriched with CCSL (2% w/v) and *A. niger* spore inoculum of 1×10^6 spores mL^{-1} maintained at 30°C . Mass transfer was achieved by a single 3 bladed pitched impeller (30 x 20 mm) at an agitation speed regulated between 350-700 rpm depending on the level of the dissolved oxygen (DO) detected in the bioreactor. The DO was maintained above the critical point ($>20\%$) with air and oxygen supplied through a sparger and the levels regulated by a rotameter at 0.5 vessel volume per min (0.5 VVM). Sampling was done through a port installed with a $0.2 \mu\text{m}$ air filter connected to a syringe (Fig. 4.4) for suction into JA 20 centrifuge bottles. Foaming was controlled by addition of 0.1% (v/v) of antifoam A (30% aqueous emulsion with emulsifiers, Sigma). Excess foam was collected in a foam trap.



Figure 4.4: Sampling of *Aspergillus niger* biomass and AbfB enzyme supernatant for determination of enzyme activity and biomass concentration.

4.1.7 Standard biomass characterisation

Mycelia (Fig. 4.3a) and pellets (4.3b) harvested from the bioreactor and terminated shake flasks cultures were observed under a microscope (100 x magnification) prior to filtering through Mira cloth mesh (Fig. 4.5). The biomass was washed with deionised water (dH₂O) before being transferred into pre-weighed aluminium foils for drying in an oven set at 60°C until a constant weight was obtained. The biomass concentration was calculated as an average dry weight of biomass (dry wt) per unit volume of the culture (g L⁻¹).

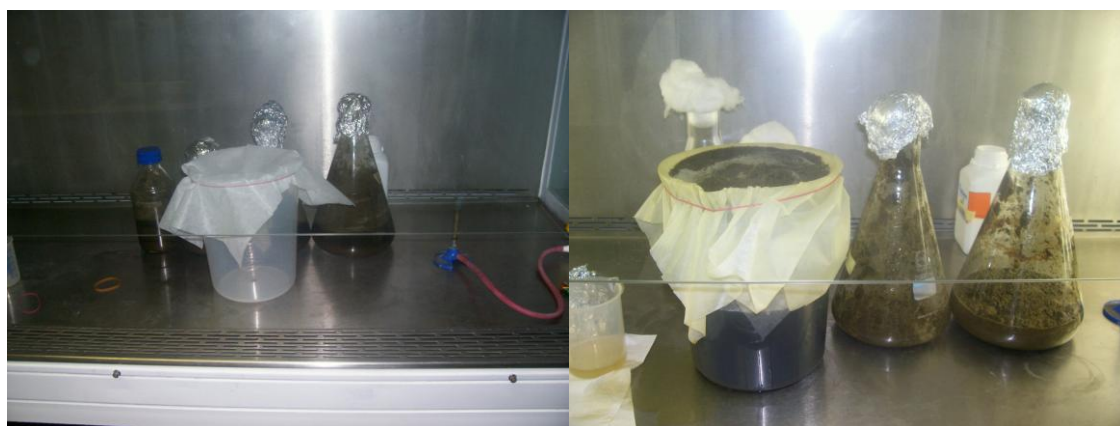


Figure 4.5: Recovery of α -L-arabinofuranosidase from fermentation cultures by filtering the *Aspergillus niger* biomass through mira cloth.

4.1.8 Partial purification of recombinant AbfB and characterisation

The culture supernatant obtained from the bioreactor fermentation was filtered through Mira cloth (Fig. 4.5). The filtrate was centrifuged (12 000 rpm for 10 min at 4°C), the resulting supernatant filtered through 0.2 μ m filters, concentrated using an

Amicon system (Amicon Division, USA) with a molecular weight cut-off (MWCO) of 10 kDa followed by lyophilisation in a Virtis freeze dryer. The concentrated enzyme supernatant was subjected to ammonium sulphate fractionation at percentage saturation of 20% and 80% (Roche, 2007) while mixing at 200-250 rpm for 4 h at 4°C. The protein mixture was centrifuged at 12 000 rpm for 1 h at 4°C. The pellet obtained at the desired saturation level was re-suspended in 5 mL of Mill-Q water and dialysed against 2 L of 10 mM acetate buffer (pH 5.0) at 4°C overnight. The desalted concentrated enzyme supernatant was subjected to a single step partial purification in a gel filtration chromatography (ÄKTA purifier system, Amersham Pharmacia Biotech) using a Superdex 75 HR 10/30 size exclusion column. Elution took place in 0.05 M acetate buffer pH 4.0 containing 0.4 M NaCl at a flow rate of 0.5 mL min⁻¹. Fractions with AbfB activity were pooled together and concentrated using the Amicon system before molecular and kinetic analysis.

4.1.9 AbfB substrate specificity and dependency and recyclability

Crude and partially purified forms of recombinant AbfB were assessed for activity against *p*NPA and polymeric xylan substrates. The polymeric xylan substrates tested included: oat spelt xylan (10:15:75, arabinose:glucose:xylose, Sigma), low viscosity wheat arabinoxylan (37:61:2, arabinose:xylose:other sugars, Megazyme), corn fibre xylan (30% arabinose) donated by Dr. Shin Li, NRL, Preoria, IL, U.S.A), debranched arabinan (sugar beet), (88:4:2:6, Arabinose:Galactose:Rhaminose:Galacturonic acid, Megazyme), larchwood arabinogalactan (15:85 Arabinose:Galactose, Megazyme) and xylans extracted from bagasse, pine (*Pinus patula*) and bamboo (*Bambusidae balcooa*) (Chapter 3). A solution containing 1% (w/v) was prepared for each of the xylan substrates. The substrates with limited solubility in water were prepared according to De Wet et al. (2008). In brief, the reaction mixture with a final volume of 5 mL, contained 2.5 mL of the xylan substrate, 1.5 mL 0.05 M citrate buffer (pH 5.0), 1 mL AbfB (approx. 18.0 nkat mL⁻¹). The reaction was performed in a water bath set at 40°C for 16-24 h. Termination of the reaction was achieved by cooling the test tubes on ice. The enzymatic hydrolysates (200 µL) were diluted 5 times and centrifuged at 10 000 rpm for 5 min at 4°C followed by filtration using filters with 0.22 µm pore size and stored at -20°C. The sugar analysis was performed using a high pH anion exchange chromatography coupled with pulsed electrochemical detection (HPAEC-PAD) (Dionex) equipped with a gradient pump GP 50, a CarbpacTM PA 10

(4 mm X 250 mm) column and electrochemical detector (ED40) for pulsed amperometric detection (PAD). The column was eluted with helium degassed 250 mM NaOH and Mill-Q water in 1.5:98.5 ratio at elution rate of 1 mL min⁻¹). Quantity of the respective sugars was determined from standard curve plot of chromatogram curve area for the respective analytical grade sugars (arabinose, rhaminose, galactose, glucose, mannose and xylose). The hydrolysate obtained from the recombinant AbfB hydrolysis of the natural substrates was assayed for residual enzyme activity in order to determine recyclability.

4.2 RESULTS

4.2.1 Extracellular production of AbfB by *A. niger* D15 [*abfB*]

The recombinant AbfB cultivated in shake flasks on standard 2 x MM media had a volumetric activity of $\approx 10.0 \text{ nkat mL}^{-1}$ (Fig. 4.6 b). The recombinant AbfB cultivated in shake flasks containing 2xMM enriched with 1%, 2% and 10% CCSL were by the second day 1.8, 2.2 and 2.6 times respectively of the volumetric activity obtained in 2xMM media (Fig. 4.6b). The maximum biomass concentration of *A. niger* grown on CCSL enriched medium in the bioreactor was as high as 33.44 g.L^{-1} on dry weight (DW) basis (Fig. 4.6 c). The AbfB produced in the bioreactor on medium enriched with 2% CCSL had a maximum volumetric activity of approximately 8.0 nkat mL^{-1} (0.450 U mL^{-1}) (Fig. 4.6c). The AbfB production level increased with an increase in biomass concentration (Fig. 4.6c). The decline in biomass growth corresponded to the decrease in sugar concentration (Fig. 4.6c). The recombinant AbfB biomass specific activity at maximum biomass concentration assayed after 48 h of incubation in the 10 L bioreactor was $227.20 \text{ nkat g}^{-1}$ on DW basis, whereas, the biomass concentration assayed at harvest (after 144 h of incubation) was $366.30 \text{ nkat g}^{-1}$ on DW basis (Fig. 4.6 c).

The protein yield for the AbfB produced on 2xMM media enriched with 2% CCSL in the 10 L bioreactor was approximately 0.167 g L^{-1} at maximum biomass concentration of 32 g L^{-1} (DW) and at harvest the protein yield was 0.45 g L^{-1} (Table 4.2). The biomass specific activity of the recombinant AbfB produced in shake flasks cultures on normal 2xMM media was $305.40 \text{ nkat g}^{-1}$ (DW) at harvest whereas, the biomass specific activity of the recombinant AbfB produced in the shake flasks on 2xMM media enriched with 2% CCSL was $207.50 \text{ nkat g}^{-1}$ (DW) (Table 4.2). The recombinant AbfB protein concentration in standard 2xMM media at harvest was 0.12 g L^{-1} , whereas that in shake flask containing 2xMM media enriched with 2% CCSL was 0.20 g L^{-1} (Table 4.2). In crude form, the specific activity of the recombinant AbfB produced in the bioreactor was approximately 2.9 nkat mg^{-1} (Table 4.3). The *A. niger* morphology changed in the bioreactor from pellets to an extensive network of mycelia. The mycelia cell lysis was evident after 144 h of incubation, which corresponded to the fall in biomass concentration but the enzyme activity remained stable (Fig.4.6d).

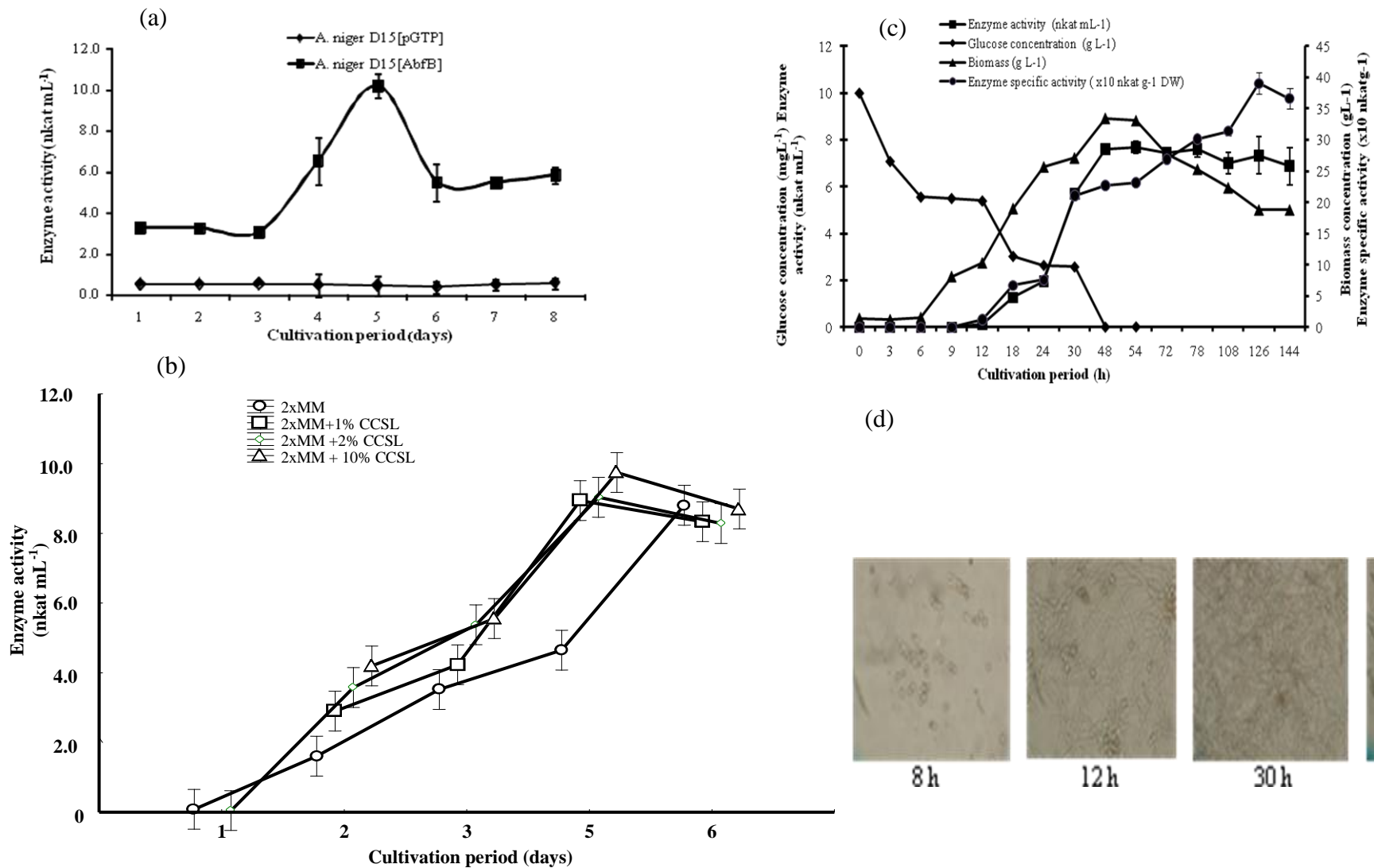


Figure 4.6: Production characteristics of AbfB by recombinant *A. niger* D15[abfB] cultivated in shake flasks in (a) pellet form, (b) AbfB activities against *p*NPA in standard 2xMM media (std) in comparison with activities when produced on 1% concentrate corn steep liquor enriched media (2xMM with 1%ccsl), 2% concentrate corn steep liquor enriched media (2xMM with 2%ccsl) and 10% concentrate corn steep liquor enriched media (2xMM with 10% ccsl), (c) production of AbfB in bioreactor (mycelial morphology) indicating extracellular AbfB activity against *p*-PNPA (nkat mL⁻¹), biomass concentration (g L⁻¹), substrate consumption (mg L⁻¹) and AbfB biomass specific activity (nkatg⁻¹DW) and (d) morphological changes at different time intervals (h) during cultivation in a bioreactor 10 L BIOFLO 110. Vertical bars denote standard deviation.

Table 4.2: Protein and biomass yield of α -L-Arabinofuranosidase in different cultivation media

Media	Protein yield (g L ⁻¹)	Biomass concentration (g L ⁻¹ dry weight)	Enzyme biomass specific activity (nkat g ⁻¹ dry weight)
2xMM (shake flask)	0.12	30.0 ± 5.0	305.40 ± 4.6
2x MM + 2%CCSL (Shake flask)	0.20	51.0 ± 2.0	207.50 ± 11.7
2x MM + 2% CCSL (10 L bioreactor at harvest)	0.16	18.79 ± 0.0.	366.30 ± 1.62
2 x MM + 2% CCSL (Fermenter at maximum biomass concentration)	0.45	33.44 ± 0.0	227.20 ± 0.14

Table 4.3: Specific activities of crude and partially purified α -L-arabinofuranosidase with polymeric xylan substrate specificity

Enzyme	Specific activity (nkat mg ⁻¹)	Specific activity (U mg ⁻¹)	pNPA (mM)	Purity	Reference
<i>A. niger</i> (AbfB)	48	2.9	5	Crude	This study
<i>A. niger</i> (AbfB)	45.3	2.7	5	Partially purified in ammonium sulphate	This study
<i>A. niger</i> B	43.32	2.6	2.5	Crude	De Ioannes et al., 2000
<i>A. niger</i> AraB	2450	147	3.7	Purified	Rombouts et al. 1988
<i>A. terreus</i> Ara	1880	113	2	Purified	Luonteri et al., 1998
<i>T. reesei</i> Ara	1760	106	2	Purified	Luonteri et al., 1998
<i>A. nidulans</i>	383	23.5	ns	Purified	Gielkens et al., 1999
<i>A. kawachii</i> AKAbf54	478	28.7	10	Purified	Koseki et al. 2006

4.2.2 Optimal and dependence on temperature and pH

The recombinant AbfB produced by *A. niger* D15 displayed an optimal temperature of 40°C and demonstrated stability at temperatures of up to 60°C for at least 30 min (Figs. 4.7a and c). The AbfB was stable when incubated at 40°C for 1 h but lost 95% of the activity within 5 min when incubated at 80°C (Fig. 4.7c). The optimal pH of AbfB ranged from pH 3.0 to pH 5.0 and was stable over pH 3.0 to pH 6.0 (Figs. 4.7b and d).

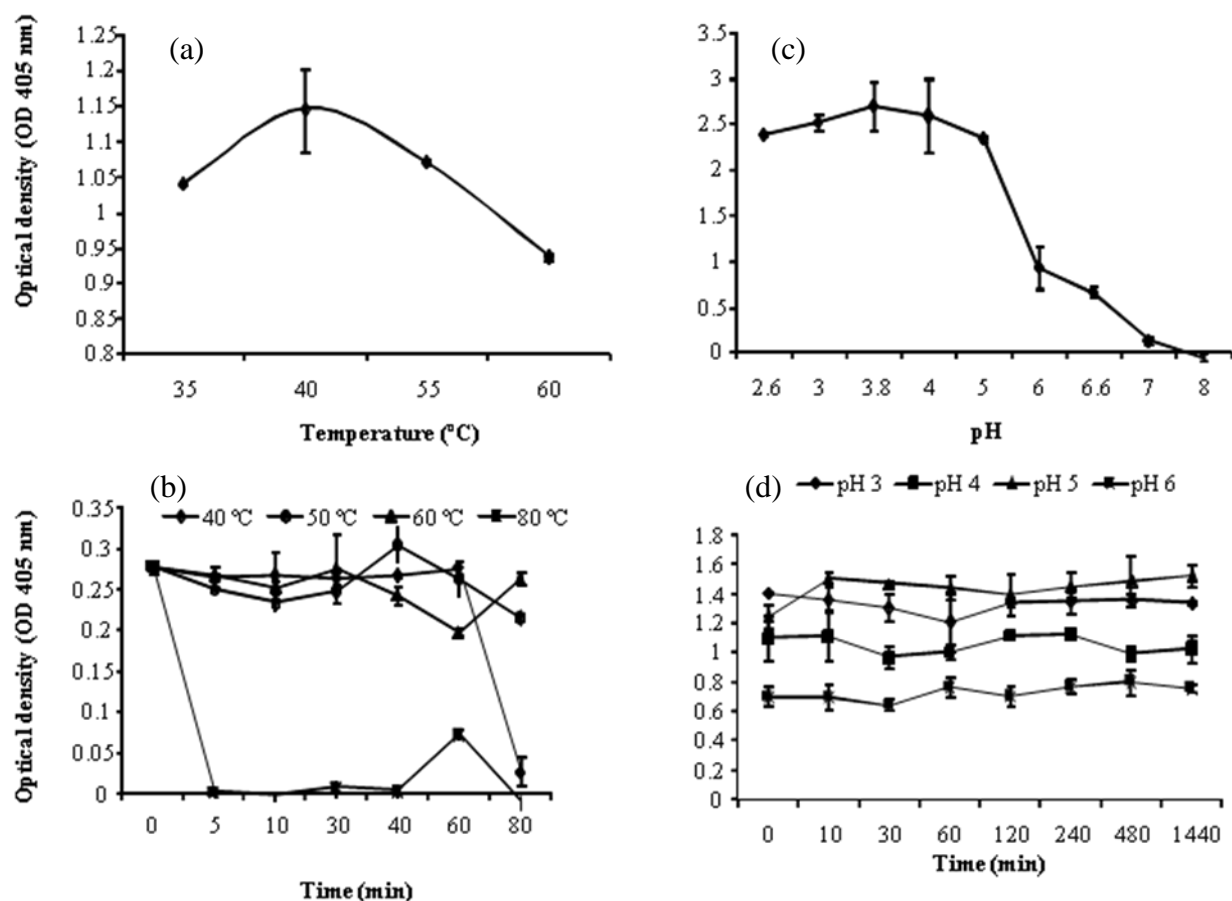


Figure 4.7: Optimal (a) temperature, (b) pH of recombinant α -L-arabinofuranosidase (AbfB) activity against pNPA and (c) stability of AbfB when incubated at 40, 50, 60 and 80 $^{\circ}$ C, and (d) at pH 3.0, pH 4.0, pH 5.0 and pH 6.0 without substrate. Assays were conducted under standard AbfB assay conditions. Vertical bars are standard deviations.

4.2.3 Molecular characteristics

The molecular weight and isoelectric point (pI) of the AbfB enzyme estimated from *abfB* gene sequence using DNAMAN sequence analysis software were 52.5 kDa and pI 4.04 whereas the estimated molecular weight from silver stained (Biorad) 10% SDS-PAGE was 67 kDa (Fig. 4.8). Multiple protein species were visualised in the 10% SDS-PAGE of AbfB produced in bioreactor on 2% CCSL enriched 2xMM medium and the AbfB partially purified using ÄKTA system. Single protein species was present in the SDS-PAGE of the AbfB produced in shake flasks both on 2xMM and CCSL enriched media (Fig. 4.8 i).

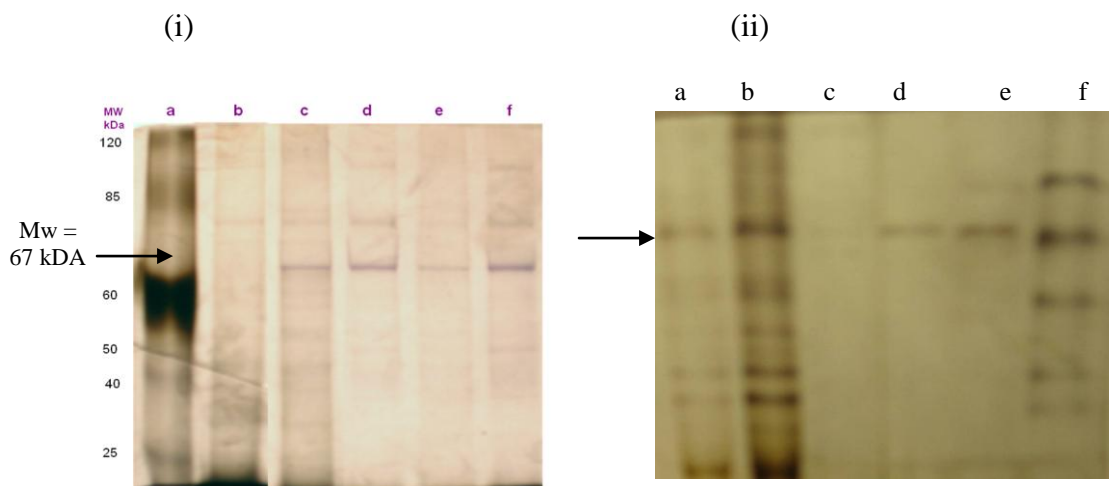


Figure 4.8: Molecular weight estimation in (i) silver stained (Biorad) 10% Sodium dodecyl sulphate - polyacrylamide gel electrophoresis (SDS-PAGE) of crude alpha L- arabinofuranosidase produced by recombinant *A. niger*). Lanes (a) bench mark pre-stained protein marker (Invitrogen Cat no 10748010); (b) AbfB negative control; (c) and (e) AbfB supernatant diluted 10x; and (d) and (f) AbfB supernatant 5x diluted and (ii) comparison of silver stained (Biorad) 10% SDS-Page of crude alpha L- arabinofuranosidase profile in lane (a) produced in CCSL enriched medium in a 10 L bioreactor diluted 20x, (b) CCSL enriched medium in bioreactor at 10x dilution (c) in standard medium in shake flask at 20x dilution, (d) in standard medium in shake flask at 10x dilution, (e) AkTA partially purified AbfB produced in CCSL enriched medium in fermenters at 20x dilution, and (f) AkTA partially purified AbfB produced in CCSL enriched medium in fermenters at 10x dilution. The gels were prepared according to Laemmli (1970).

4.2.4 Substrate dependency and substrate specificity

The dependency on *p*NPA concentration of the activity of crude recombinant AbfB is presented in Figure 4.9a for recombinant AbfB produced in shake flasks on 2xMM medium and Figure 4.9c for recombinant AbfB produced on 2% CCSL enriched medium. The initial specific activity of the crude recombinant AbfB against *p*NPA increased linearly with increase in *p*NPA concentration, reaching the maximum velocity (V_{\max}) between 30 and 40 mM *p*NPA (Fig. 4.9a and c). The K_m values for the recombinant AbfB produced in 2xMM medium and 2xMM medium enriched with 2% CCSL were estimated at *p*NPA concentration of 9.0 and 7.0 mM respectively (Figs. 4.9b and d).

The AbfB released arabinose from low viscosity wheat arabinoxylan, corn fibre xylan, debranched arabinan and larchwood arabinogalactan xylan at approximately 20.0, 2.0, 7.0 and 7.0% of the available arabinose (Fig. 4.10a). The action of the AbfB on the substrates was reflected by visible precipitation of the xylan polymers (Fig. 4.10b). The recombinant AbfB released 20% of available arabinose from the oat spelt arabinoxylan, 9% from arabinoglucuronoxylan extracted from bagasse, 5% each from arabinoglucuronoxylan extracted from bamboo and *P. patula* and about 4% from H_2O_2 bleached bagasse (Fig. 4.10c).

4.2.5 Storability and recyclability

The relative residual activity against *p*NPA of AbfB, which was initially stored at 4°C, increased by 13% within 24 h of storage at 26, 30 and 37°C (Fig. 4.11a). The AbfB activity at the three storage temperatures remained stable up to 72 h where no significant differences ($p<0.05$) in the AbfB activities were observed for the entire storage period. The residual AbfB activity after hydrolysis of H₂O₂ bleached bagasse xylan and xylans extracted from bagasse, bamboo and *P. patula* was more than 95% after 16 h of hydrolysis (Fig. 4.11b).

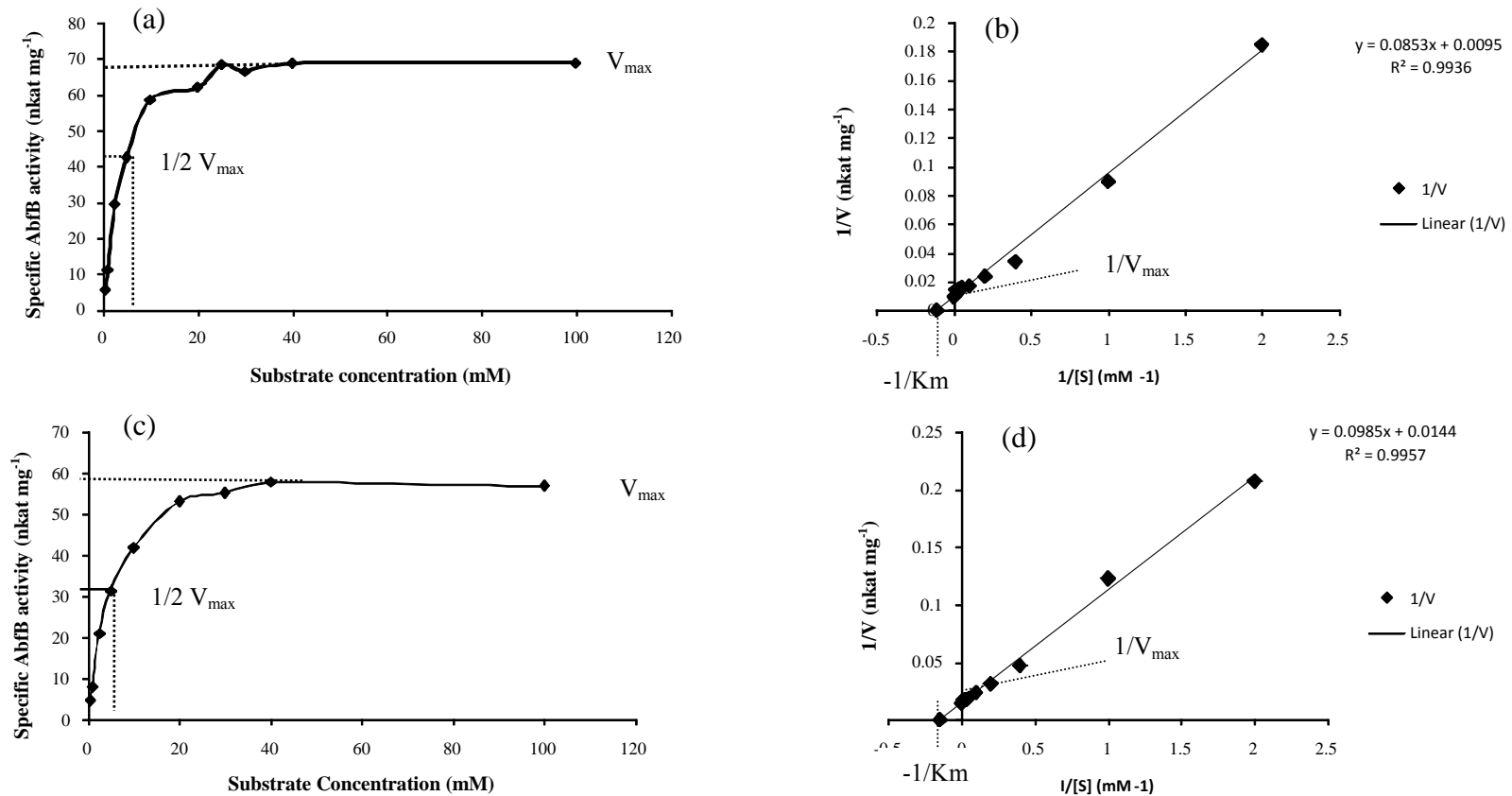


Figure 4.9: Saturation kinetics showing effect of *p*NPA concentration on the rate of hydrolysis by (a) recombinant AbfB produced in shake flask on 2xMM media, (b) double reciprocal (line weaver- Burk) plot, ($1/V$) and *p*NPA concentration ($1/[S]$), of initial specific activity for AbfB produced in shake flasks on 2xMM media, (c) effect of *p*NPA concentration on the rate of hydrolysis by AbfB produced in shake flask on 2xMM media enriched with 2% CCSL and (d) double reciprocal (line weaver- Burk) plot, ($1/V$) and *p*NPA concentration ($1/[S]$), of initial specific activity for AbfB produced in shake flasks on 2xMM media enriched with 2% CCSL. Dotted lines are showing positions for V_{max} or K_m values.

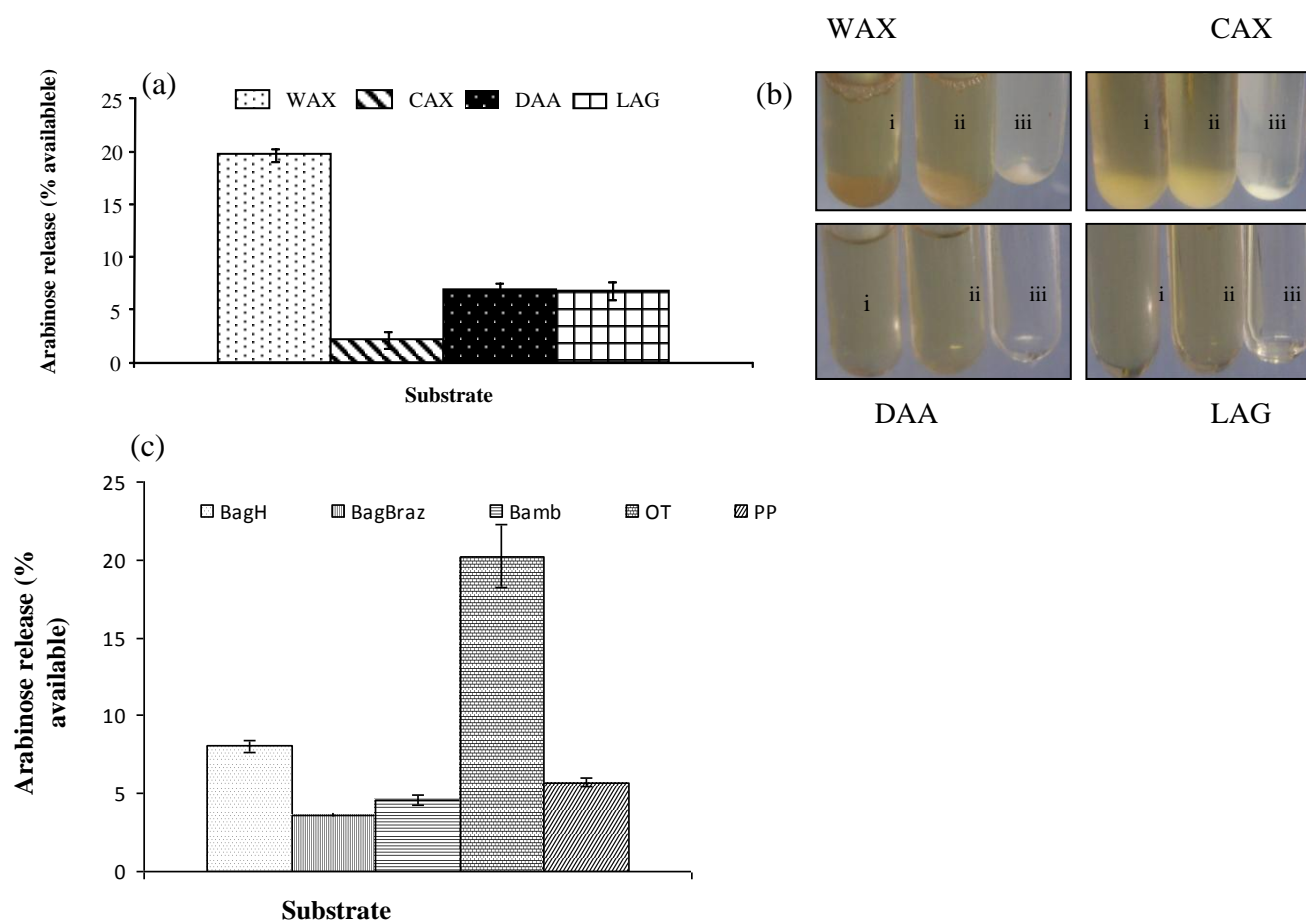


Figure 4.10: Removal of arabinose by recombinant AbfB from (a) low viscosity wheat xylan (WAX), corn fibre xylan (CAX), debrached arabinan (DAA) and larchwood arabinogalactan LAG), (b) Effect of removal of arabinose from WAX (top left), CAX (top right), DAA (bottom left) and LAG (bottom right). Numbers i and ii represent duplicates of AbfB treated sample and iii represents control sample (untreated). (c) Arabinose release from bagasse xylan extracted by the Hoiije method (Bag H), H₂O₂ bleached bagasse xylan (BagBraz), bamboo xylan (Bamb), oat spelt xylan (OT) and *P. patula* xylan (PP). Recombinant AbfB hydrolysis was performed at 40 °C and pH 5.0 for 16 h.

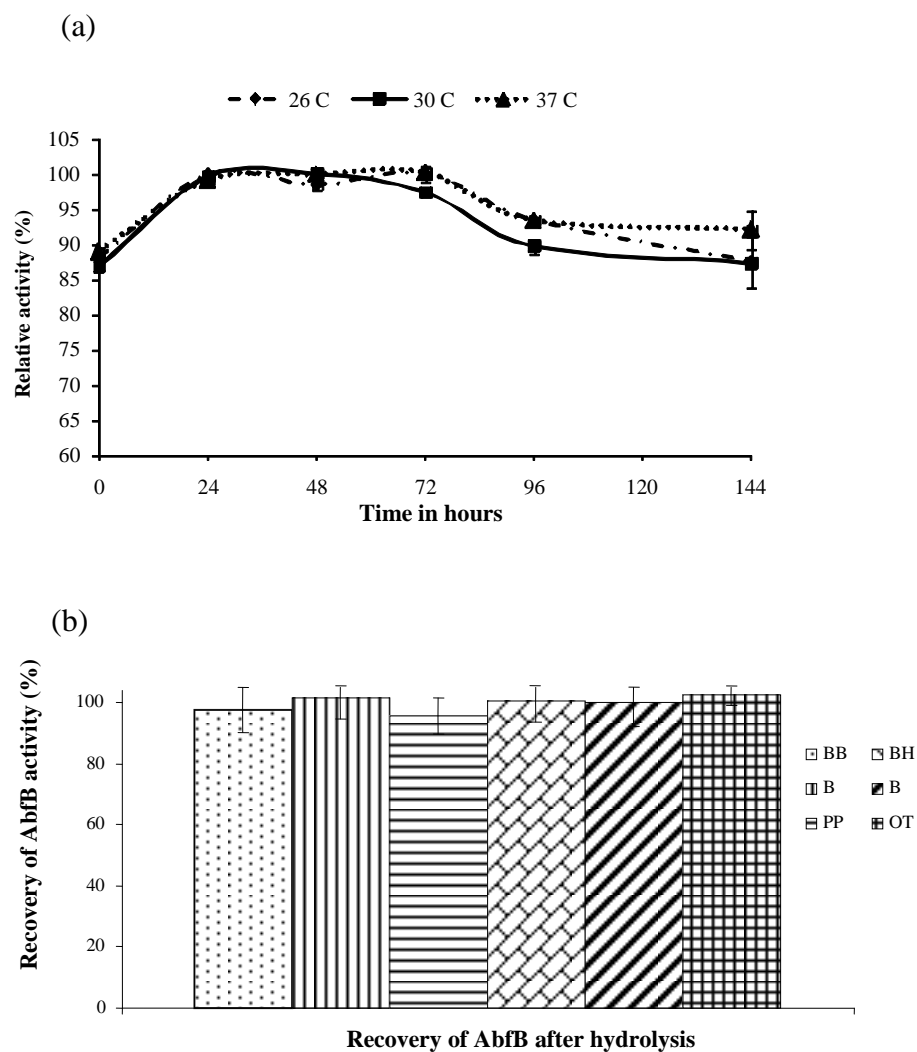


Figure 4.11: (a) Stability of recombinant α -L-arabinofuranosidase at varying storage temperatures and (b) recyclability after hydrolysis of H_2O_2 bleached bagasse xylan (BB), bagasse xylan extracted by the Hoiye method (BH), bagasse xylan extracted by the Lopez method (BL), bamboo xylan (BM), *P. patula* xylan (PP) and oat spelt xylan (OT). The recombinant AbfB hydrolysis was performed at 40°C, pH 5.0 for 24 h.

4.3 DISCUSSION

4.3.1 Production characteristics of recombinant AbfB

The *abfB* gene from *A. niger* was overexpressed in the *A. niger* D15 protease-deficient strain under the transcriptional control of glyceraldehyde-3-phosphate dehydrogenase promoter (*gpd_p*) and glucoamylase terminator (*glaA_T*). The recombinant *A. niger* D15 secreted extracellularly recombinant AbfB in both the standard 2xMM media and 2xMM media enriched with 2% CCSL, which was free of xylanase activity. Therefore, the transformed *A. niger* D15 strain presents an improved microbial system for production of the recombinant AbfB enzyme that can be used in crude form for selective hydrolysis of water soluble xylans substituted with arabinose side chains. The silver stained 10% SDS-PAGE clearly displayed prominent single protein species bands of the recombinant AbfB, which was produced in shake flasks on both standard 2xMM and 2xMM with 2% CCSL enriched medium (Fig. 4.8a). Although, multiple proteins species bands were visible in the silver stained 10% SDS-PAGE of the recombinant AbfB produced in the 10 L bioreactor on 2xMM media that was enriched with the 2% CCSL, the protein band for the recombinant AbfB was the most prominent (Fig. 4.8b). It is possible that the observed multiple protein species bands on the silver stained 10% SDS-PAGE originated from intracellular proteins or other protein compounds released upon breakage or lysing of the mycelium in the bioreactor (Fig. 4.6d (iv)) as the biomass was aging (Fig. 4.6c). These results suggest that the modification of the transcriptional control for the constitutive expression of the *abfB* gene in the *A. niger* D15 protease-deficient strain was effective in enabling selective extracellular secretion of recombinant AbfB free of xylanase activity..

The recombinant AbfB was secreted in media in the shake flasks (Fig. 4.6a) and bioreactor (Fig. 4.6b) cultures with volumetric activities against *p*NPA substrate of more than 20 times the volumetric activities of recombinant AbfB of 0.020 U mL⁻¹ when it was previously expressed in *S. cerevisiae* (Crous et al., 1996). The *A. niger* microbial systems are considered prolific producers of functional industrial enzymes because of the inherent higher capacity for protein secretion compared to the *S. cerevisiae* based system. Such characteristic is probably because of highly efficient protein posttranscriptional modification processes (Verdoes et al., 1997; Conesa et al., 2001; Punt et al., 2002). The specific activity of the recombinant AbfB against *p*NPA substrate was 2.9 U mg⁻¹, which was higher than the specific activity of 2.7 U mg⁻¹

observed in its partially purified state (Table 4.3). The results showed that the recombinant *A. niger* microbial system had increased capacity to express the recombinant AbfB extracellularly compared to wild type *A. niger* strains and other microbial expression systems reported in literature (Table 4.3). The results showed that the specific activity of the recombinant AbfB against the *pNPA* substrate both in crude and partially purified form, were higher than the specific activities of 2.6 U mg^{-1} of crude *A. niger* B arabinofuranosidase reported by De Ioannes et al. (2000) and that of 1.07 U mg^{-1} for the recombinant AbfB expressed by *Fusarium oxysporum* f. sp. *dianthi* (Carlos et al., 2004) (Table 4.3). The enhanced secretion could be attributed to the expression of the recombinant AbfB that was under the transcriptional control of a strong *gpd_P* promoter. A similar effect was observed with the expression of homologous feruloyl esterase B in protease deficient *A. niger* strain microbial system when expressed under a similar promoter (Levasseur et al., 2004). However, the recombinant AbfB protein yields obtained in this study ($0.12\text{-}0.45 \text{ g L}^{-1}$) were less than the potential protein yields of up to 30 g L^{-1} reported for *A. niger* industrial expression systems for production of other proteins (Dunn-Coleman et al., 1992; Kinghorn and Unkles, 1994; Conesa et al., 2001; Punt et al., 2001). Therefore, further improvements of the *A. niger* microbial systems might be necessary to increase the protein yields.

The secretion of the recombinant AbfB by the *A. niger* D15 protease-deficient strain was growth associated (Fig. 4.6c). The results showed that the increase in the recombinant AbfB volumetric activity coincided with the increase in the biomass growth such that the maximum recombinant AbfB activity of 8.0 nkat mL^{-1} was achieved immediately after attaining the maximum biomass growth of 33.44 g L^{-1} between 36 and 48 h of cultivation. Furthermore, in the shake flask cultures, the recombinant AbfB protein concentration almost doubled (from 0.12 mg mL^{-1} to 0.2 mg mL^{-1}) with enrichment of the standard 2xMM fermentation media with 2% (w/v) CCSL as an additional source of nitrogen (Table 4.2). Growth associated production of enzymes is characteristic of enzymes produced constitutively in *A. niger* (Gweyn, 1992; Shuler and Kargi, 2002). Such behaviour was also observed during production of α -glucoamylase by *A. niger* (Pedersen et al., 2000). Therefore, the production of the recombinant AbfB can be enhanced by maintaining high biomass production. The results show that enrichment of the standard 2xMM cultivation medium with the CCSL accelerated to the secretion of the recombinant AbfB by the transformed *A.*

niger D15 protease-deficient strain. The transformed *A. niger* D15 protease-deficient strain cultivated in 1%, 2% and 10% CCSL enriched media were by the second day of incubation, secreting recombinant AbfB with volumetric activities against *p*NPA that were 1.8, 2.2 and 2.6 times respectively, the volumetric activity of the recombinant AbfB secreted in the standard 2xMM media (Fig. 4.6c). The recombinant AbfB activity in 2 and 10% CCSL enriched media had already reached the maximum volumetric activity of 10 nkat mL⁻¹ on the 5th day of incubation, which was one day earlier than in the 2xMM media (Fig. 4.6c). Therefore, the addition of the CCSL to the 2xMM standard cultivation media was a viable strategy for promoting both the biomass growth and the volumetric activity of the recombinant AbfB. The CCSL is an industrial waste and as such presents cost effective way of supplying additional nitrogen for accelerated and increased production of the recombinant AbfB. The recombinant AbfB protein yield per unit biomass (DW) for bioreactor fermentation performed in 2xMM cultivation media enriched with 2% (w/v) CCSL was 366.30 nkat g⁻¹ (DW) which was higher than that of 207.5 nkat g⁻¹ (DW) for fermentation performed in shake flasks in similar media (Table 4.2). Gurlal et al. (2006) reported similar association of increased biomass growth (35 g L⁻¹) with increase in recombinant mannase secretion by *A. niger* D15 protease deficient strain of up to 32% by enriching the standard 2xMM cultivation media with the CCSL under transcriptional control of *gpd* promoter. However, extreme biomass growth could present a challenging task in downstream processing (Dun-Colemann, 1992; Harvey and McNeil, 1994).

4.3.2 Mode of AbfB enzyme production

Improved quantities of the recombinant AbfB could be produced by scaling up the production of the recombinant *A. niger* microbial system developed in this study. The results showed that the extracellular activity of the recombinant AbfB was stable in the 10 L bioreactor cultures (Fig. 4.6c) compared to shake flask fermentation cultures, in which the activity sharply declined immediately after reaching the maximum (Fig. 4.6a). Similarly, the differences between the recombinant AbfB protein yields per unit biomass (DW) realised in the 10 L bioreactor and the shake flasks could possibly be attributed to (1) limited working volume, (2) lack of adequate control of the growing conditions such as the pH and dissolved oxygen in the shake flasks cultures and (3) formation of pellet morphology. Such factors are known to limit dissolved oxygen

transfer at the solid-liquid interface and transport within the pellets (Kennedy and Krouse, 1999; Hille et al., 2005) compared to the conditions in the 10 L bioreactor fermentation cultures. Therefore, the results suggest that it is possible to efficiently increase specific yield of the recombinant AbfB by either scaling up the fermentation system and/or producing the recombinant AbfB in mycelia form under controlled cultivation conditions (Fig. 4.3b). Therefore, production of the recombinant AbfB in higher quantities would be more suitable if performed in large scale bioreactor fermentations than in the shake flasks. However, there is still need for further optimisation of the production process in order to improve the recombinant AbfB yields to match those reported in literature.

4.3.3 Molecular and physical properties of the recombinant AbfB

The recombinant AbfB displayed molecular and physical characteristics similar to most α -L-arabinofuranosidases belonging to the glycosyl hydrolase (GH) family 54. The molecular mass (MW) of 67 kDa of the recombinant AbfB estimated on the 10% SDS-PAGE (Fig. 4.8a) was similar to that obtained by Veen et al. (1991), which falls within the range of molecular masses (49-70 kDa) described by other workers (Rombouts et al., 1988; De Ioannes et al., 2000; Koseki et al., 2000; Fritz et al., 2008; De Wet et al., 2008). However, the MW of 67 kDa observed on SDS-PAGE was greater than the MW of 52.4 kDa calculated from the gene sequence using the DNAMAN computer software. The difference between the observed and calculated AbfB molecular masses could in part be attributed to possible post translational modification such as glycosylation, which in this case could be estimated to be 29%. The glycosylation is required for the enzyme to attain full activity and stability upon secretion (Gweyn, 1992; Conesa et al., 2001).

The recombinant AbfB can be utilised optimally at a broader temperature range (40-55°C) (Fig. 4.7a) and under a wide range of acidic conditions (pH 3.0-pH 6.0) (Fig. 4.7b). The molecular and physical characteristics of the AbfB are in agreement with properties reported for α -L-arabinofuranosidases belonging to glycoside hydrolase (GH) family 54 http://afmb.cnrs-mrs.fr/CAZY/GH_54.htm (De Ioannes et al., 2000; Kaneko et al., 2006; De Wet et al., 2008). Therefore, the recombinant AbfB has characteristics of a robust and flexible enzyme technology for selective xylan hydrolysis. The results showed that the recombinant AbfB was stored in its crude form without detectable loss of activity for over 24 months at 4°C. Furthermore, from

the 4°C storage temperature, the enzyme was stable for more than 144 h (6 days) at 26-37°C (Fig. 4.11a) and during hydrolysis of xylans performed at 40°C for 16-24 h at pH 5.0 (Fig. 4.11b). Therefore, it was possible to recycle more than 95% of the recombinant AbfB activity against *p*NPA after catalysing release of arabinose from polymeric xylan substrates (Fig. 4.11b). The observed effect is typical of enzymes that do not stay bound to their substrates after the catalytic reactions (Van Beilen and Li, 2002). However, the activity of the recombinant AbfB was reduced by over 50% at temperatures of > 60°C. A loss of over 95% of the activity was observed within five minutes of incubation at 80°C (Fig 4.7d). Therefore, recombinant AbfB temperature and pH stability suggest that direct application of the enzyme in lignocelluloses processing such as the kraft pulp and paper making processes would require some adjustment of the temperature and pH. However, the stability in storage and recyclability after xylan hydrolysis of the recombinant AbfB would particularly reduce the cost of production and handling. Furthermore, the recyclability of the enzyme after xylan hydrolysis implies less contamination of the enzymes of the xylan product. Therefore, the recombinant AbfB presents a robust and flexible technology that can be used for selective xylan hydrolysis and be compatible for use in processes where high purity standards are required.

4.3.4 AbfB kinetics and substrate specificity

The recombinant AbfB, produced both in the standard and CCSL enriched media displayed Michaelis-Menten (saturation) kinetic properties that reached equilibrium at 40 mM *p*NPA. The saturation curves provided estimated K_m values between 6-10 mM upon catalysis of *p*NPA of varying concentration (Fig. 4.9). The observed K_m values are within the values reported by Tajana et al. (1992) who reported K_m values of 10-12.5 mM for two α -L-arabinofuranosidases obtained from *Streptomyces diastaticus*. However, these values are higher for K_m values of 4.8×10^{-4} M reported for fungal arabinofuranosidases from *A. niger* by Rombouts et al. (1988). Although the K_m value is an intrinsic parameter that should remain constant for a specific enzyme (Shuler and Kargi, 2002), the results from this study showed that the K_m values were different for the same AbfB when produced in 2xMM medium and in 2xMM medium enriched with 2% CCSL (Fig. 4.9). This observation is in agreement with Shuler and Kargi (2002) who indicated that similar enzymes are likely to have different K_m values (thus possessing different affinity levels to the same substrate) if subjected to different

temperature or pH conditions and when secreted by different organisms. A low K_m suggests that the enzyme has high affinity towards the substrate and the value corresponds to the substrate concentration that would give 50% of the maximal forward velocity (V_{max}) (Shuler and Kargi, 2002). Therefore, the K_m values obtained for the AbfB suggests lower affinity towards the *p*NPA compared to the corresponding AbfB reported by Rombouts et al. (1988).

The AbfB presents a potential technology for selective removal of arabinose side chains from a broader spectrum of lignocellulosic materials without degrading the main xylan chains, which confirmed that the recombinant AbfB lacked β -xylanase activity. The recombinant AbfB catalysed liberation of α -1,2- and α -1,3-bonded arabinose side chains from low viscosity wheat xylan and corn fibre xylan substituted with arabinose and MeGlcA side chains (Fig. 4.10c). In addition, the AbfB was capable of removing 7% of α -1,3-bound arabinose from larchwood arabinogalactan and α -1,5-bonded arabinose from debranched arabinan (Fig. 4.10a). Such characteristic is an indication of the broad substrate specificity of the GH family 54 AbfBs (De Ioannes et al., 2000). The ability of the AbfB to remove α -1,5-bonded arabinose in this study is also in agreement with studies by Luonteri et al. (1998) whereby 5% of available arabinose was released from linear beet α -1,5-bound arabinose, but contradicts the results on lack of action by the recombinant AbfB from *A. pullulans* on similar substrate reported by De Wet et al. (2008). Therefore, it appears substrate specificity of the AbfB may have changed. However, the removal of the arabinose from the low viscosity wheat arabinoxylan and corn fibre xylan caused visible precipitation (Fig. 4.10b), indicating the ability of recombinant AbfB for use in insolubilising water soluble xylans. Therefore, the recombinant AbfB has potential use for production of insoluble xylan hydrogels from xylans.

The AbfB had greater affinity towards arabinoxylans compared to arabinoglucuronoxylans as such higher degree of arabinose removal was obtained from oat spelt than from arabinoglucuronoxylans extracted from bagasse, bamboo and *P. patula* (Chapter 3) and from H_2O_2 bleached bagasse xylans. The results have shown that 20% of available arabinose was released from oat spelt arabinoxylan compared to 9% released from arabinoglucuronoxylans extracted from bagasse by the Hoijs method (Fig. 3.10c). The relatively limited release of the arabinose from

substrates such as arabinoglucuronoxylans of bagasse and *P. patula* origin could be attributed to limited accessibility of the AbfB to the side chains due to steric hindrance from α -D-MeGlcA side chains. Therefore, synergistic application of the recombinant AbfB and of pure α -D-glucuronidase (with polymeric substrate specificity and xylanase free) might improve the release of arabinose.

4.4 CONCLUSION

An *Aspergillus niger* D15[abfB] strain selectively secreted α -L- arabinofuranosidase (AbfB) with improved purity and crude enzyme specific activity of 2.9 U mg^{-1} under the transcriptional control of the glyceraldehyde-3-phosphate dehydrogenase promoter (*gpd_P*) and glucoamylase terminator (*glaA_T*). The AbfB secretion was growth associated and as such the biomass growth and protein yield per unit biomass were improved by cultivating the recombinant *A. niger* in 2xMM media enriched with 2% concentrated corn steep liquor. The AbfB was stable under cultivation, storage and application conditions at temperatures between 30-60°C and pH 3.0-6.0 providing the possibility of multiple applications from a single AbfB dosage. The secreted recombinant α -L-arabinofuranosidase (AbfB) displayed polymeric xylan substrate specificity, thus having the ability to remove arabinose side chains from polymeric xylans and cause the xylans to precipitate. The extracellular overexpression of the recombinant AbfB in relatively purer and larger quantities than wild type strain, presents a novel biological tool for diversifying functional properties of xylans from various feedstocks as novel speciality additives, coatings and hydrogels encapsulation matrices.

Chapter 5: Selective enzymatic hydrolysis to reduce the solubility of water soluble xylans

ABSTRACT

The ability of the recombinant α -L-arabinofuranosidase (AbfB) of *Aspergillus niger* and purified α -D-glucuronidase (AguA) of *Schizophyllum commune* to reduce the solubility of water soluble arabinoglucuronoxylans extracted from sugarcane (*Saccharum officinarum* L) bagasse, bamboo (*Bambusidae balcooa*) and *Pinus patula* and glucuronoxylan from *Eucalyptus grandis* found in South Africa, was assessed. The recombinant AbfB precipitated the water soluble arabinoxylan extracted from oat spelt and water soluble arabinoglucuronoxylans extracted from bagasse and bamboo but not extracted from *P. patula*. On the other hand, the purified AguA could only precipitate glucuronoxylan extracted from birch but not those extracted from *E. grandis* and arabinoglucuronoxylans extracted from bagasse, bamboo and *P. patula*. The selective removal of the arabinose increased the viscosity of bagasse, bamboo and oat spelt xylans. However, with time, the arabinose side chain removal exhibited a negative correlation with xylan viscosity. The viscosity of oat spelt xylan reached a minimum of 2.03 mPa.s at a shear rate of 10.3 s^{-1} after the removal of the arabinose side groups by the recombinant AbfB at xylan specific dosage of $400.0 \text{ nkat g}^{-1}$ for 9.0 h at 45.8°C . There was a synergetic increase in the removal of arabinose side chains of 22% from bagasse, 33% from bamboo xylan during combined application of the recombinant AbfB and purified AguA. However, there was a synergetic decrease of 13% in the removal of MeGlcA side chains from bamboo xylan. Therefore, the recombinant AbfB and purified AguA offer a potential novel technique for decreasing solubility and formation of hydrogels from bamboo, *E. grandis* and bagasse polymeric xylans.

5.0 INTRODUCTION

Xylan is one of the major polysaccharides present in lignocellulosic materials, which has potential to be an abundant renewable source of biodegradable and biocompatible. There is growing demand for production of with such properties from natural sources owing to increased environmental consciousness and high cost of producing synthetic polymers from fossil fuel. Biodegradable and biocompatible are needed in a wide range of industries including pharmaceutical, biomedical, food, pulp and paper making and cosmetics (Dumitriu, 2002). Xylans particularly needed, are those that possess increased adhesive power and have hydrogel forming properties, which can be used as bonding and coating agents and for production of biomaterials and biocomposites (Cole, 1992; Henriksson and Gatenholm, 2001; Gröndahl and Gatenholm, 2005; Westbye et al., 2006). Insoluble xylans are particularly important for their strong hydrogel/film forming and adhesive properties (Dumitriu, 2002; Lindblad et al., 2001). Such applications would provide the xylans with high economic value compared to the current practice of incinerating xylans containing waste streams for the recovery of energy or land filling that happens at most pulp mills.

The industrial application of the xylans is currently far less than that of cellulose and starch owing to higher water solubility of the xylans as a consequence of low degree of polymerisation (DP), lack of crystallinity and high degree of substitution on the main chain (Gröndahl and Gatenholm, 2005; Spiridon and Popa, 2005; Ebringerová et al., 2005). Furthermore, the appropriate techniques for reducing the solubility of xylans are not well developed compared to those of cellulose and starch. Meanwhile, production of from cellulose and starch is faced with competition from other applications including food and speciality chemical industries. Therefore, the development of a technology for altering the physicochemical properties of the xylans, especially reducing their water solubility, would transform the xylans into suitable alternatives to cellulose and starch in many industrial applications.

Xylan is structurally similar to cellulose but instead of the β -D-glucopyranose backbone, the backbone consists mainly of 1,4- β -linked xylopyranose backbone to which side groups such as acetyl, α -L-arabinofuranoside (arabinose), 4-O-methyl

glucuronic acid (MeGlcA) or both arabinose and MeGlcA are attached (Sjöström, 1993; Dekker, 1989). However, the distribution pattern and degree of substitution by these xylan side groups may be different in plant species and in parts of the same species (Ebringerová and Heinze, 2000; Wilkie, 1979). The presence of the side groups such as arabinose, MeGlcA and acetyl on the xylan backbone cause steric hindrances that make the xylan highly soluble in water (Brice and Morrison, 1982; Viikari et al., 1993; Izydorczyk and Biliaderis, 1995; Ebringerová and Heinze, 2000; Gröndahl et al., 2003; Finnie et al., 2006). Therefore, the removal of the side chains constitutes an important process for precipitating the xylans that would subsequently diversify their functional properties to match those of cellulose and starch. Moreover, the removed side chain sugar residues constitute an additional value added by-product stream in lignocelluloses processing because of their unique medicinal properties (Seri et al., 1996; Silva et al., 1998; Ebringerová and Heinze, 2000; Ebringerová et al., 2002).

Precipitation of xylans is a common phenomenon during the late stages of kraft pulping, which is associated with cleavage of the side groups by alkali hydrolysis upon loss of kraft cook alkalinity at high temperature (Danielsson, 2007). Such processes increase the self association and re-adsorption of the xylans onto the pulp fibres (Sjöström, 1993; Danielsson, 2007). However, the kraft conditions degrade the xylans to organic acids, which are disposed off in the black liquor waste streams as such, contributing substantially to environmental pollution. Side chain removing enzymes offer a less polluting method for precipitation of the xylans because they are highly selective for the removal of the side chains in contrast to the random degradation of the xylans that occur during the kraft cooking. The side chain removing enzymes such as recombinant α -L-arabinofuranosidase (EC 3.2.1.55) (AbfB) (Chapter 4) and purified α -D-glucuronidase (EC 3.2.1.139/131) (AguA) (Tenkanen and Siika-aho, 2000) are responsible for the selective cleavage of arabinose and MeGlcA side chains respectively, from water soluble xylans. Furthermore, these AbfBs and AguAs could be applied in synergy to enhance complete degradation of substituted oligomeric xylan into xylose residues generated by the xylan main chain degrading enzymes (Biely, 2003).

The use of the side chain removing enzymes for production of insoluble xylans as speciality additives and coating material in industrial applications has not been studied systematically. The status quo has partly been attributed to the limited availability of the water soluble polymeric xylans and the side chain removing enzymes with polymeric xylan substrate specificity. Although the ability of the AbfB in selective removal of the arabinose side chains from xylans has been known for over decades, the enzyme is not readily available in pure form and in recombinant systems. However, it was possible to produce extracellular AbfB with polymeric xylan substrate specificity that was free of detectable activity for the main chain degrading enzymes (xylanase activity) by using recombinant *A. niger* (D15) protease deficient strain (Chapter 4). The AguA with ability to remove MeGlcA from polymeric xylan substrates is an enzyme that has been isolated and purified from a few microbial organisms including *Schizophyllum commune* (Khandke, 1989; Tenkanen and Siika-aho, 2000) and from *Pichia stipitis* (Ryabova et al., 2009), but has not been cloned or expressed in recombinant systems. Most of the microbial systems produce the AbfBs and AguAs in association with xylanase activity that degrade the main xylan chain as well (Biely, 2003). Even though the AbfB and AguA might have the polymeric xylan substrate specificity, their direct use for selective removal of side chains to reduce solubility of water soluble polymeric xylans is limited without expensive purification procedures. Therefore, both the recombinant AbfB and purified AguA with polymeric xylan substrate specificities are highly novel enzymes that could be used to reduce the solubility of polymeric xylans extracted from different grass and wood sources in a customised manner.

The purpose of this study was to assess the ability of the recombinant AbfB of *A. niger* and purified AguA of *S. commune*, individually and in combination for controlled reduction of solubility of arabinoglucuronoxylans extracted from sugarcane (*Saccharum officinarum* L) bagasse, bamboo (*Bambusidae balcooa*) and *Pinus patula* and glucuronoxylan from *Eucalyptus grandis* found in South Africa (Chapter 3). The recombinant AbfB and the purified AguA were applied to selectively remove arabinose and MeGlcA side chains respectively, from the xylans under varying hydrolysis time, temperature levels and enzyme xylan specific dosages. Oat spelt, H₂O₂ bleached bagasse and birch xylans and *E. grandis* xylan gel were included in the assessment for reference purposes.

5.1 MATERIALS AND METHODS

5.1.1 Materials

The arabinoglucuronoxylans for the study were extracted from sugarcane (*S. officinarum* L) bagasse, bamboo (*B. balcooa*) and *P. patula*); and glucuronoxylan from *E. grandis* extracted using methods adapted from Höije et al. (2005) and De Lopez et al. (1996) (Chapter 3). The H₂O₂ bleached bagasse xylan was generously donated by Prof. A.M.F. Milagres, University of São Paulo, Lorena, Brazil. The oat spelt xylan (X-0627) with a sugar composition of 10:15:75 (arabinose: glucose: xylose) was purchased from Sigma. The *E. grandis* xylan gel extracted from pulp fibres was generously donated by Dr Arlene Bayley of SAPPI Technology Centre, Pretoria, South Africa. The detailed descriptions of the xylans in the study are presented in Table 5.1.

The crude α -L- arabinofuranosidase (AbfB) with volumetric activity of 18.0 nkat mL⁻¹ on *p*-nitrophenyl arabinofuranoside (*p*NPA) was produced in house by use of recombinant *A. niger* D15 (Chapter 4). The purified α -D-glucuronidase (AguA) with specific activity of 300 nkat mg⁻¹ isolated from wild type *S. commune* (VTT-D-88362-ATCC 38548) was generously donated by Prof. Matti Siika-aho of VTT Biotechnology institute in Finland. The recombinant AbfB and purified AguA enzyme aliquots were stored at 4°C for the entire duration of the study.

5.1.2 Preparation of xylans

Xylan solutions (1% w/v) were prepared in de-ionized water (dH₂O). The xylans that showed limited solubility in water was prepared by first dissolving in ethanol and subsequently heating the solution to 80°C according to the protocol described by De Wet et al. (2008). Xylan solutions were made in bulk and stored in vials at -20°C.

5.1.3 Selective enzymatic hydrolysis of polymeric xylans

The selective removal of arabinose was achieved by treating the xylans with recombinant AbfB in reaction mixtures (5 mL) containing 2.5 mL soluble xylan, 1.0 mL enzyme supernatant and 1.5 mL 0.05 M citrate buffer pH 5.0. The hydrolysis was performed at 40°C for 16 h. The removal of MeGlcA side chains was done by treating xylan solutions (1% w/v) with the purified AguA at a dosage level of 9 000 nkat g⁻¹_{substrate} in 5 mL reaction mixtures consisting of 2.5 mL of the xylan solution, which

were marked up to 5 mL with 0.05 M acetate buffer, pH 4.8. The reactions proceeded for 16 h at 40°C. Synergetic selective removal of both arabinose and glucuronic side chains was performed by treating the arabinoglucuronoxylan solutions (1% w/v) with a cocktail of recombinant AbfB and purified AguA in 1000 µL reaction mixtures containing 500 µL of the arabinoglucuronoxylan and 500 µL of a cocktail of recombinant AbfB dosage of 540.0 nkat g⁻¹_{substrate} and purified AguA dosage of 9 000 nkat g⁻¹_{substrate} in 0.05 M acetate buffer pH 4.8. The reactions were performed in a water bath set at 40°C for 24 h. The synergetic effects of the AbfB and AguA were calculated based on the difference between the amount of the arabinose or MeGlcA released due to individual enzyme action and combined enzyme action.

5.1.4 Effect of enzymatic selective hydrolysis on viscosity, degree of precipitation and aggregation of polymeric xylans

Evaluation of the effect of enzymatic removal of side chains under varying hydrolysis time, temperature and enzyme xylan specific dosages, on the xylan solubility properties, in particular viscosity was done only with the recombinant AbfB. The influence of hydrolysis time, temperature and enzyme xylan specific dosage on viscosity of polymeric xylan was measured using rheometer (MCR501) at 25°C. The precipitation and aggregation, of the xylan hydrogels was assessed by visual inspection (photographs taken) and was quantified by determining the content of the residual xylose present in solution using phenol-sulphuric assay for total sugar analysis (Dubois, et al., 1956).

The effect of varying AguA specific xylan dosage on the removal of MeGlcA side group from polymeric xylans and the subsequent change in solubility properties, in particular precipitation, were assessed at three xylan specific dosage levels, 2500, 5000, 8000 nkat g⁻¹_{substrate} in 0.5 mL. The reaction mixture consisted of 0.05 M acetate buffer pH 4.8, enzyme supernatant and 450 µL of 1% (w/v) birch xylan. The reactions were performed at 40°C for 24 h.

5.1.5 Determining sugar profile of enzymatic xylan hydrolysates

Arabinose and MeGlcA side chains were analysed using high pH anion exchange chromatography coupled with pulsed electrochemical detection (HPAEC-PAD) (Dionex) that was equipped with a gradient pump GP 50, a CarboPacTM PA 10 (4 mm

X 250 mm) column and electrochemical detector (ED40). The column was eluted with helium degassed 250 mM NaOH and Milli-Q water in the following proportion 1.5:98.5. Additionally, sodium acetate (1M NaOAc) eluent was included during the analysis of MeGlcA sugars as described by Tenkanen and Siika-aho (2000). The elution rate was 1 mL min⁻¹. The data acquisition and quantification of sugar concentrations was done by the inbuilt PEAKNET software package. The L (+) - arabinose (Sigma, A3256) and D-MeGlcA (Sigma, G5269D) were used as standard sugars.

5.1.6 Statistical analysis

The effects of hydrolysis time, temperature and enzyme xylan specific dosage on the viscosity of water soluble xylans were determined using a response surface three factor Box-Behnken statistical design with 3 central points in 15 runs [Statistica 7.0 software programme, (StatSoft, Inc., 1984-2005)] as shown in Table 5.2. The statistical analyses included regression, ANOVA and desirability profiling for determining optimal values for various combinations of the hydrolysis parameters for achieving desired degree of viscosity change. The response surface plots were fitted to a second order polynomial model (Equation 5.1) in Statistica.

$$Z = \beta_0 + \beta_1 X_1 + \beta_{11} X_1^2 + \beta_2 X_2 + \beta_{22} X_2^2 + \beta_3 X_3 + \beta_{33} X_3^2 + \varepsilon \quad (5.1)$$

Where:

- Z = Viscosity (mPa.s)
- $\beta_0, \beta_1, \dots, \beta_n$ = Linear regression coefficients,
- $\beta_{11}, \dots, \beta_{nn}$ = Quadratic regression coefficients
- X_1, X_2, X_3 = Hydrolysis time, temperature and xylan specific dosage respectively
- ε = Error

Table 5.1: Description of xylan substrates for selective enzymatic removal of side chains

Xylan	Method of extraction	Type (s) of side chain	Content (% substrate dry weight)			Source
			Arabinose	MeglCA	Xylose	
Sugarcane (<i>Saccharum officinarum</i> L) Bagasse (BH)	Mild alkali (Höije)	Arabinose , MeGlcA	17.4	8.5	71	Chapter 3
Sugarcane (<i>Saccharum officinarum</i> L) Bagasse (BL)	Mild alkali (Lopéz)	Arabinose, MeGlcA	10.0	8.5	71	Chapter 3
Sugarcane (<i>Saccharum officinarum</i> L) bagasse (BB)	Mild alkali H ₂ O ₂ bleached	Arabinose, MeGlcA	10.0			Generously donated by Prof. A.M.F. Milagres, University of São Paulo, Brazil
giant bamboo (<i>Bambusidae balcoa</i>) (BM)	Mild alkali (Höije)	Arabinose, MeGlcA	10.5	11.2	79.5	Chapter 4
Eucalyptus (<i>Eucalyptus grandis</i>) (EH)	Mild alkali (Höije)	MeGlcA	0.3	12.8	82.1	Chapter 3
Eucalyptus (<i>Eucalyptus grandis</i>) (EL)	Mild alkali (Lopéz)	MeGlcA	0.3	12.8	82.1	Chapter 3
Eucalyptus (<i>Eucalyptus grandis</i>) gel from pulp (ES)	Nd	MeGlcA	nd	<3.0	>97	Generously donated by Dr. Arlene Bayley, SAPPI Technology Centre, Pretoria, RSA
Pine (<i>Pinus patula</i>) (PP)	Höije et al. (2005)	Arabinose, MeGlcA	15.5	11.50	61.3	Chapter 3
Oat spelt xylan	Nd	Arabinose	7.4 (10) ^a	-	87.0 (75) ^a	Sigma
Birch xylan	Nd	MeGlcA	-	10 (8.3) ^b	89 (89.3) ^b	Roth

Note: Nd denotes not disclosed, MeGlcA denotes 4-*O*-Methyl glucuronic Acid ; a = value in brackets determined by Sigma; b = value in brackets provided by Kormelink and Voragen (1993).

Table 5.2: Box-Behnken statistical design for determining parameters with significant effects and their optimal conditions for selective removal of arabinose and xylan viscosity.

Run	Coded variables			Natural variables		
Random order	X1	X2	X3	Time (h)	Temperature (°C)	Enzyme dose (nkat g ⁻¹)
9	0	-1	-1	8.5	30	180.0
3	-1	1	0	1	50	360.0
10	0	1	-1	8.5	50	180.0
6	1	0	-1	16	40	360.0
11	0	-1	1	8.5	30	180.0
4	1	1	0	16	50	540.0
7	-1	0	1	1	40	360.0
14	0	0	0	8.5	40	540.0
12	0	1	1	8.5	50	360.0
15	0	0	0	8.5	40	540.0
5	-1	0	-1	1	40	360.0
2	1	-1	0	16	30	180.0
13	0	0	0	8.5	40	360.0
8	1	0	1	16	40	360.0
1	-1	-1	0	1	30	540.0

***Design summary:** 3 factor Box-Behnken design, Number of factors independent variables: 3, Number of runs (cases, experiments): 15, and Number of blocks: 1

5.2 RESULTS

5.2.1 Degree of enzymatic removal of side chains from polymeric xylans

The degree of arabinose side chains as a result of individual application of the recombinant AbfB on xylans extracted from bagasse, bamboo and *P. patula* by the Hoiye method was approximately 14, 6 and 6% of the available arabinose respectively (Fig. 5.1a). Whereas the degree of liberation of MeGlcA by the individual application of the purified AguA on xylan extracted from bagasse, bamboo and *P. patula* by the Hoiye method was 2.0, 0.8 and 0.8% of the available MeGlcA respectively (Fig. 5.1b). In the combined application of the recombinant AbfB and purified AguA, the degree of arabinose removal from arabinoglucuronoxylans extracted from bagasse, bamboo and *P. patula* by the Hoiye method was approximately 17.0%, 8.0 and 7.0% of available arabinose respectively (Fig. 5.1a). The removal of MeGlcA by combined application of the recombinant AbfB and purified AguA yielded 2.1, 0.7 and 1.0% of the available MeGlcA from the bagasse, bamboo and *P. patula* arabinoglucuronoxylans respectively (Fig. 5.1b). Furthermore, the degree of MeGlcA removal as a result of the application of AguA on glucuronoxylans from birch xylan, those extracted from *E. grandis* by the Hoiye method and the *E. grandis* xylan gel extracted from pulp, was 1.2%, 0.9 and 4.0% respectively of the available MeGlcA (Fig. 5.2).

5.2.2 Effect of selective enzymatic hydrolysis on polymeric xylan viscosity

The viscosity of xylans derived from oat spelt (Figs. 5.3a), bagasse (Figs. 5.3b) and bamboo (Figs. 5.3d), selectively hydrolysed by the recombinant AbfB was higher than that of the corresponding untreated xylan fractions. Despite the removal arabinose side chains by the recombinant AbfB, there was no apparent change in the viscosity of H₂O₂ bleached bagasse and *P. patula* xylans (Figs. 5.3c and e). The recombinant AbfB selective hydrolysis of bamboo xylan resulted in an increase in viscosity (measured at shear rate of 18 s⁻¹) of between 5-34% with AbfB xylan specific dosage of up to 360.0 nkat g⁻¹_{substrate} (Fig. 5.4a).

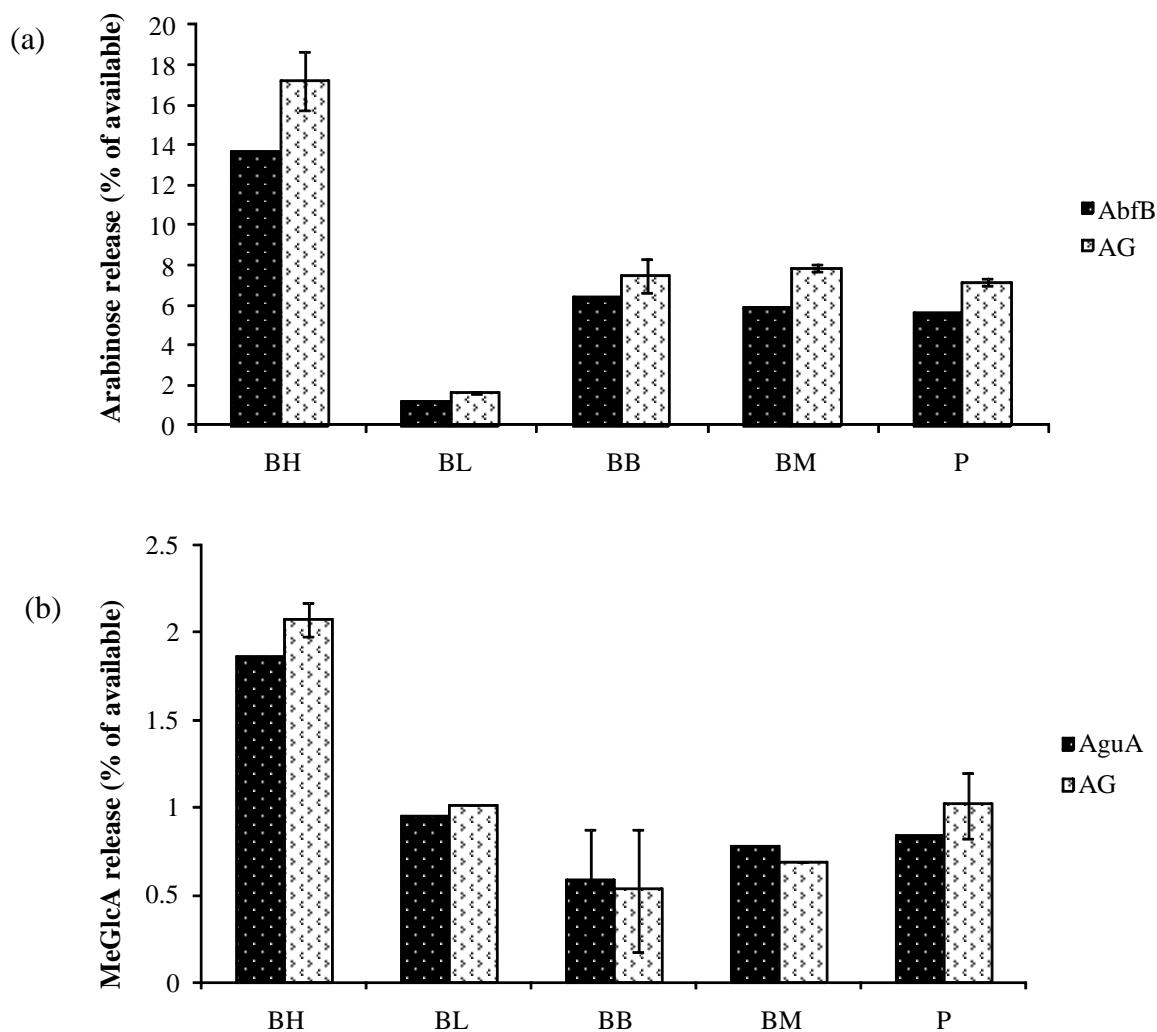


Figure 5.1: Removal of (a) arabinose and (b) MeGlcA as a result of individual and combined application of AbfB and AguA on mild alkali pre-extracted bagasse (BH), bamboo (BM), and *Pinus patula* (PP) xylan extracted using Hoije method, bagasse extracted using Lopez method (BL) and H₂O₂ bleached bagasse (BB). The arabinoglucuronoxylans were treated with recombinant AbfB xylan specific dosage = 720.0 nkat g⁻¹_{substrate}, and AguA xylan specific dose = 9000 nkat g⁻¹_{substrate} substrate, Temperature of 40°C, hydrolysis time of 24 h at pH 4.8 AguA = purified α -D-glucuronidase), AbfB = recombinant α -L-arabinofuranosidase and AG = combined enzyme preparation of recombinant AbfB and purified AguA.

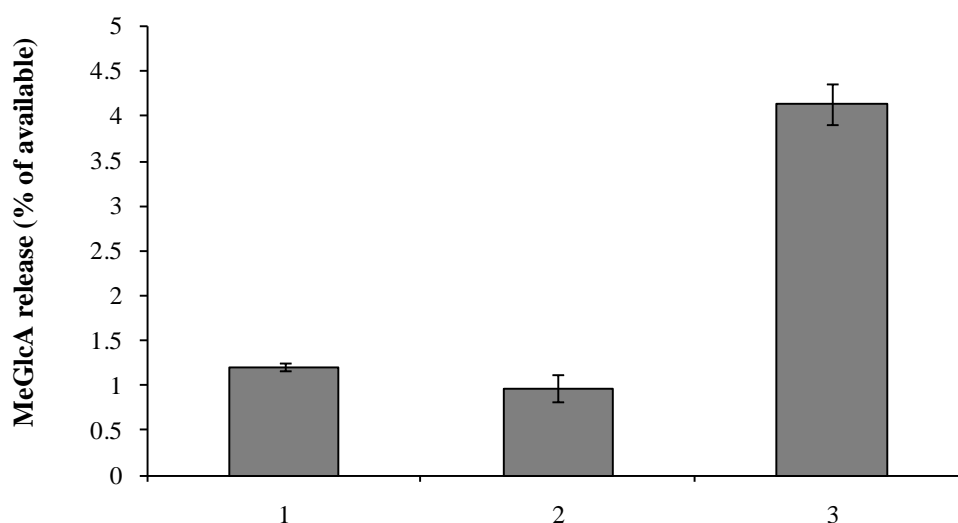


Figure 5.2: MeGlcA removal by AguA from (1) birch, (2) mild alkali pre-extracted *Eucalyptus grandis* (EH) and (3) *Eucalyptus grandis* gel extracted from pulp (ES) singly substituted with MeGlcA, with AguA xylan specific dosage of 9000 nkat g⁻¹ substrate, Temperature = 40°C and time = 16 h at pH 4.8.

Furthermore, the viscosity of oat spelt xylan solution (measured at shear rate of 18 s⁻¹) after selective hydrolysis by the recombinant AbfB at 40°C was 50% higher than the viscosity at 60°C (Fig. 5.4b). In addition, the increase in AbfB xylan specific dosage from 180.0 nkat g⁻¹ to 720.0 nkat g⁻¹ substrate increased the viscosity of the oat spelt xylan hydrolysed by the recombinant AbfB by 10% (Fig. 5.4b). However, the viscosity of the oat spelt xylan correlated negatively [$r(X, Y) = -0.544$, $p < 0.05$] with increased selective removal of arabinose by the recombinant AbfB (Fig. 5. 5).

5.2.3 Response surface plot for xylan viscosity.

The response surface plots fitted with second order polynomial for viscosity as a function of hydrolysis time, temperature and AbfB xylan specific dosage reflected a minimum viscosity of 2.03 mPa.s was reached after oat spelt xylan was hydrolysed for 9 h at 45.8°C with recombinant AbfB xylan specific dosage of 400.0 nkat⁻¹ substrate (Fig. 5.7 a-c). However, the xylan viscosity increase was observed at hydrolysis time of >10 h (Figs. 5.7a and b).

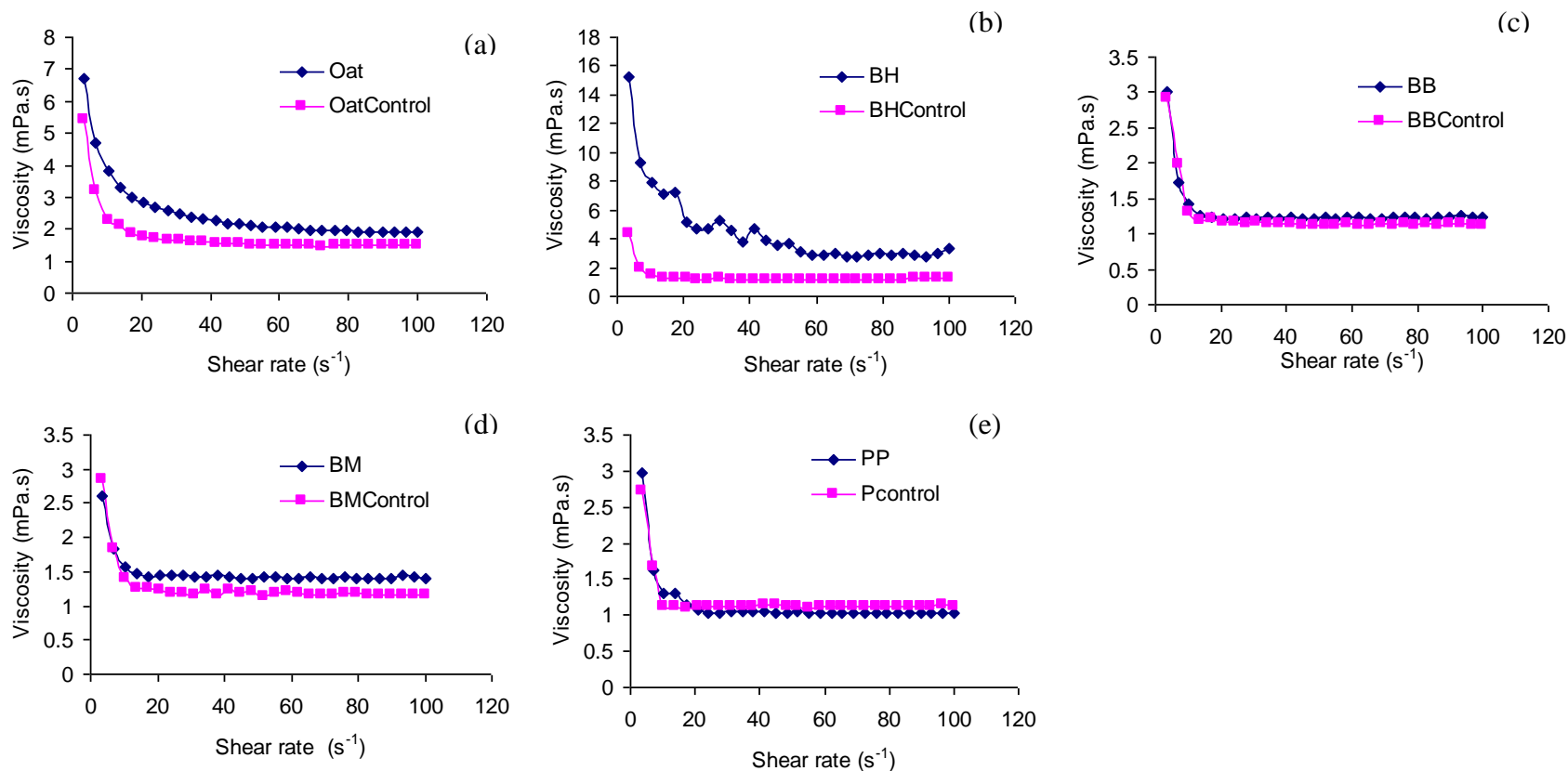


Figure 5.3: Change in viscosity of xylan with application of recombinant α -L-arabinofuranosidase (AbfB) for selective hydrolysis of (a) oat spelt xylan (b) xylan extracted from bagasse using the Hoije method (BH) (c) H₂O₂ bleached bagasse xylan (BB), (d) xylan extracted from bamboo using the Hoije method (BM), and (e) xylan extracted from *P. patula* using the Hoije method (PP). Xylan hydrolysis was performed at 40°C for 16 h with xylan specific dosage of 720.0 nkat g⁻¹ substrate.

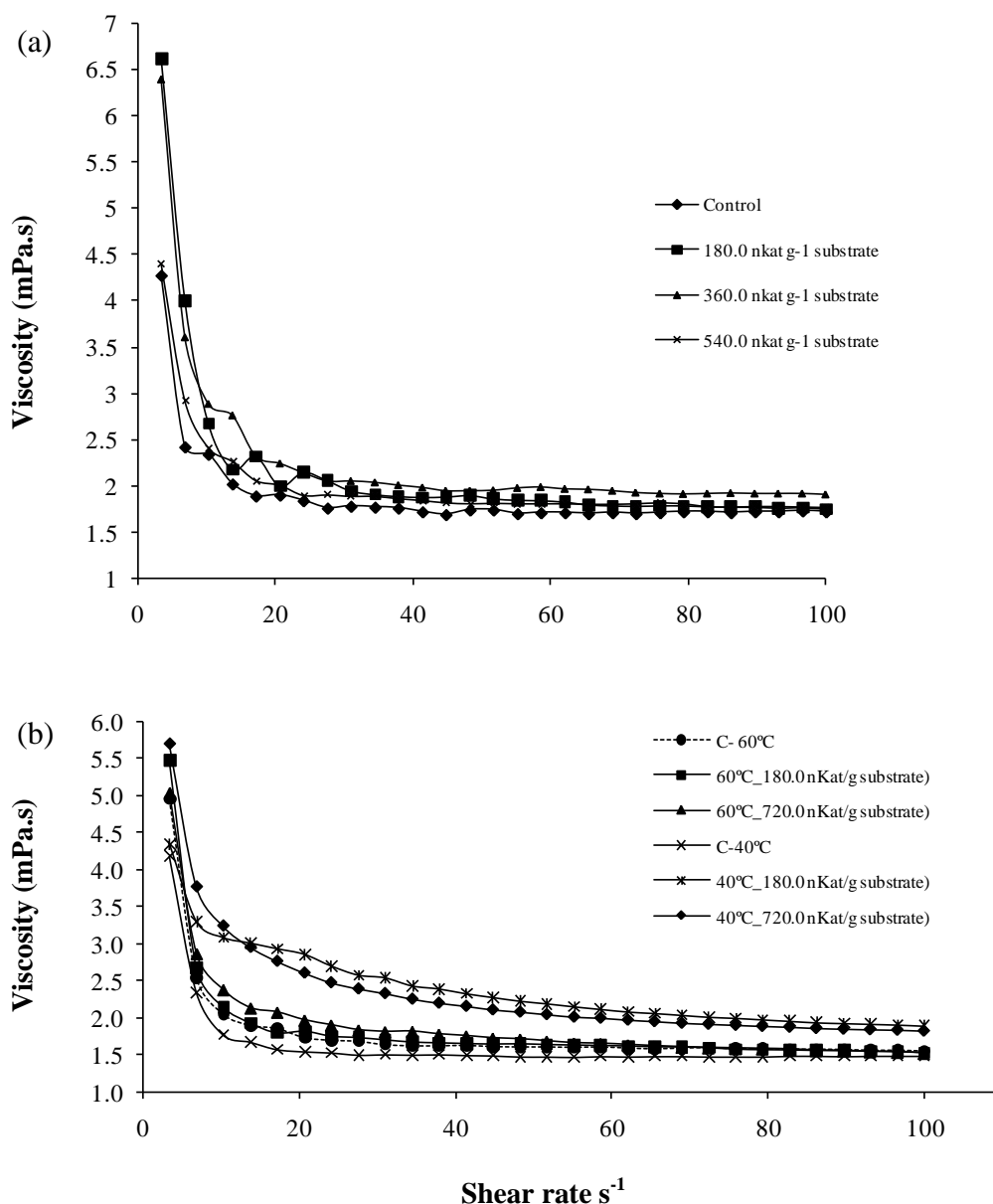


Figure 5.4: Change in viscosity of xylans after recombinant AbfB hydrolysis of (a) bamboo xylan as a function of recombinant AbfB xylan specific dosage. The recombinant AbfB hydrolysis was performed at 40°C for 1 h. (b) The change in viscosity of oat spelt xylan as a function of recombinant AbfB xylan specific dosage and temperature. The control in the legend denotes untreated sample. The AbfB hydrolyses were performed for 16 h with recombinant AbfB xylan specific dosages of 180.0 and 720.0 nkat g⁻¹ substrate.

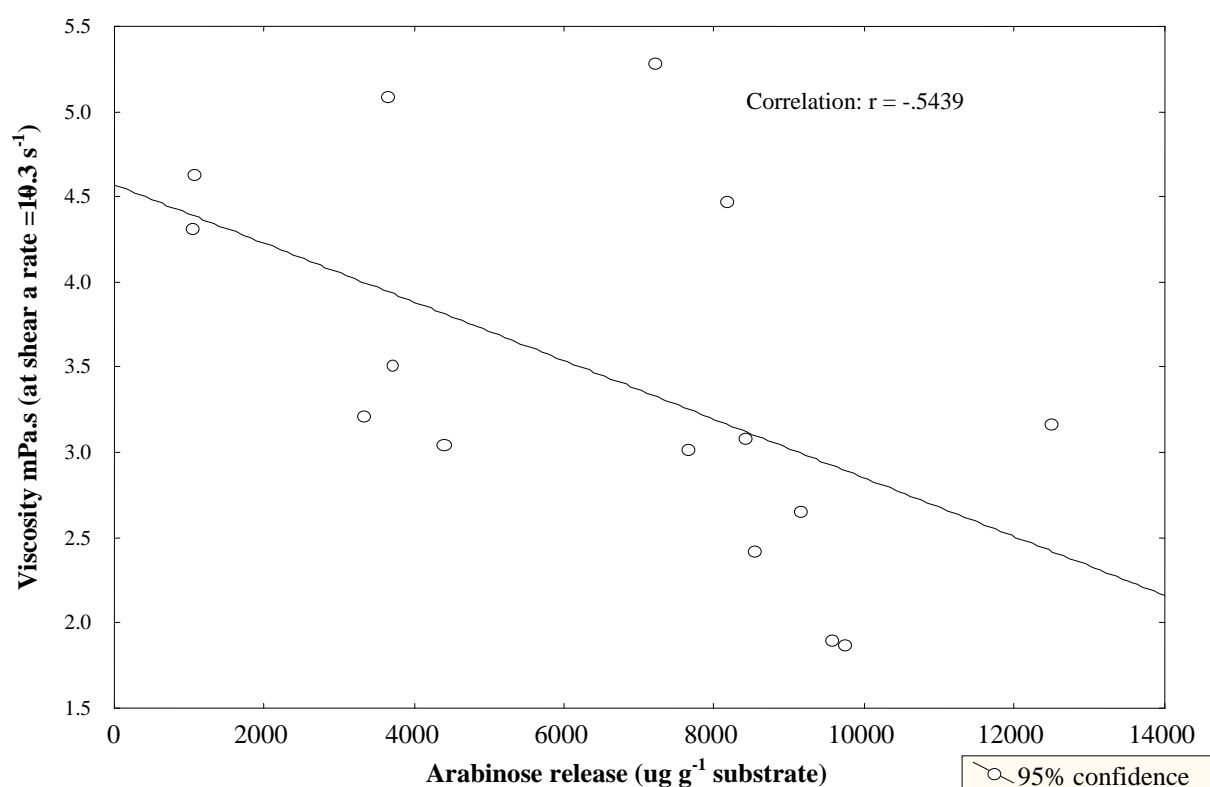


Figure 5.5: Correlation of change in viscosity of oat spelt xylan and arabinose removal. Viscosity was measured at a shear rate of 10.3s^{-1} .

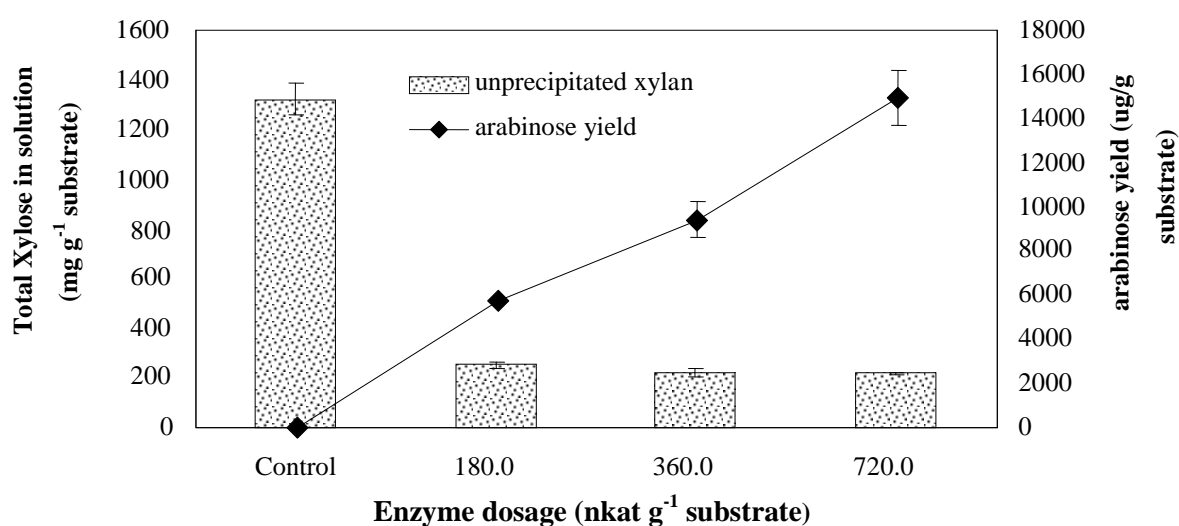


Figure 5.6: Relationship between arabinose removal and degree of precipitation after AbfB hydrolysis of oat spelt xylan with enzyme xylan specific dosages ranging from 0-720.0 nkat g⁻¹ substrate. The hydrolysis was performed at 40°C for 16 h at pH 5.0.

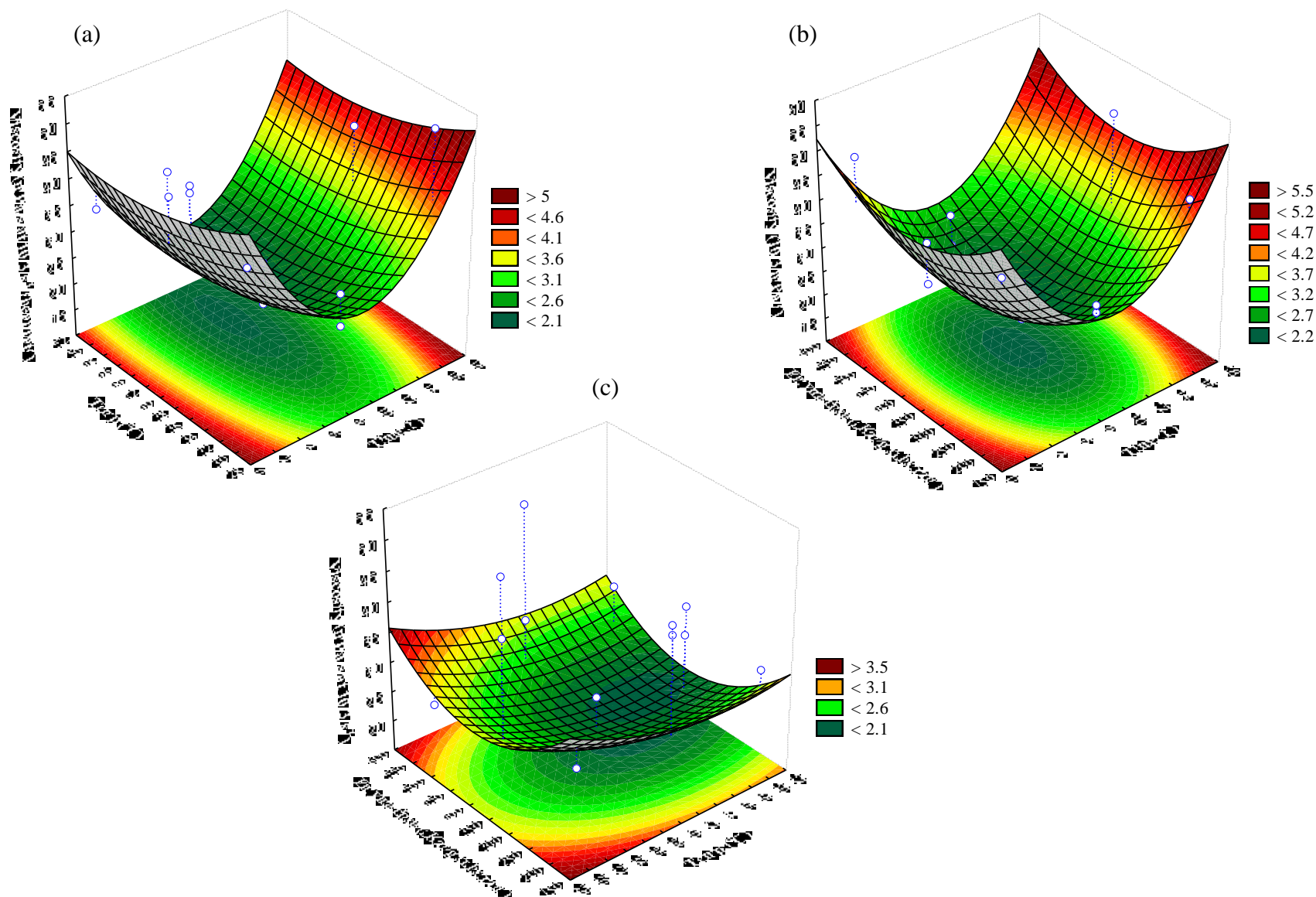


Figure 5.7: Change in viscosity of oat spelt xylan as a function of (a) time and temperature, (b) time and AbfB xylan specific dosage (nkat g^{-1}), and (c) temperature and AbfB xylan specific dosage ($\text{nkat g}^{-1}_{\text{substrate}}$). Viscosity was measured at shear rate of 10.3 s^{-1} .

5.2.4 Effect of enzymatic removal of side chains on polymeric xylan precipitation and aggregation

The precipitation of oat spelt xylan occurred after removal of $4000 \mu\text{g g}^{-1}_{\text{substrate}}$ by the recombinant AbfB, which represented 4% removal of the available arabinose (Fig. 5.6). Furthermore, the continued removal of the arabinose from the oat spelt xylan beyond $10.0 \text{ mg g}^{-1}_{\text{substrate}}$ (10% available arabinose) hardly increased the degree of precipitation of the oat spelt xylan (i.e. the amount of residual xylose in the reaction mixture levelled off before achieving maximum degree of arabinose removal from the xylans (Fig. 5.6). The removal of arabinose side chains by the recombinant AbfB from xylans extracted from bagasse by the Hoije method (Fig. 5.8b), H_2O_2 bleached bagasse xylan (Fig. 5.8c) and xylan extracted from bamboo by the Hoije method (Fig. 5.8d) resulted in precipitation and formation of visible hydrogel aggregates. The bamboo xylan precipitated hydrogel aggregates formed upon selective removal of arabinose by the recombinant AbfB (Fig. 5.8d) were in comparable amounts to those of oat spelt (Fig. 5.8a). However, the precipitation and formation of hydrogels was not visible after selective hydrolysis of arabinoglucuronoxylan extracted from *P. patula* by the recombinant AbfB (Fig. 5.8e). On the other hand visible hydrogel precipitates were evident upon selective removal of MeGlcA side chains from birch xylan by the purified AguA (Fig. 5.8f), but such xylan hydrogel precipitates were not seen in xylans extracted from bagasse, bamboo and *P. patula* after selective hydrolysis by the purified AguA (results not shown).

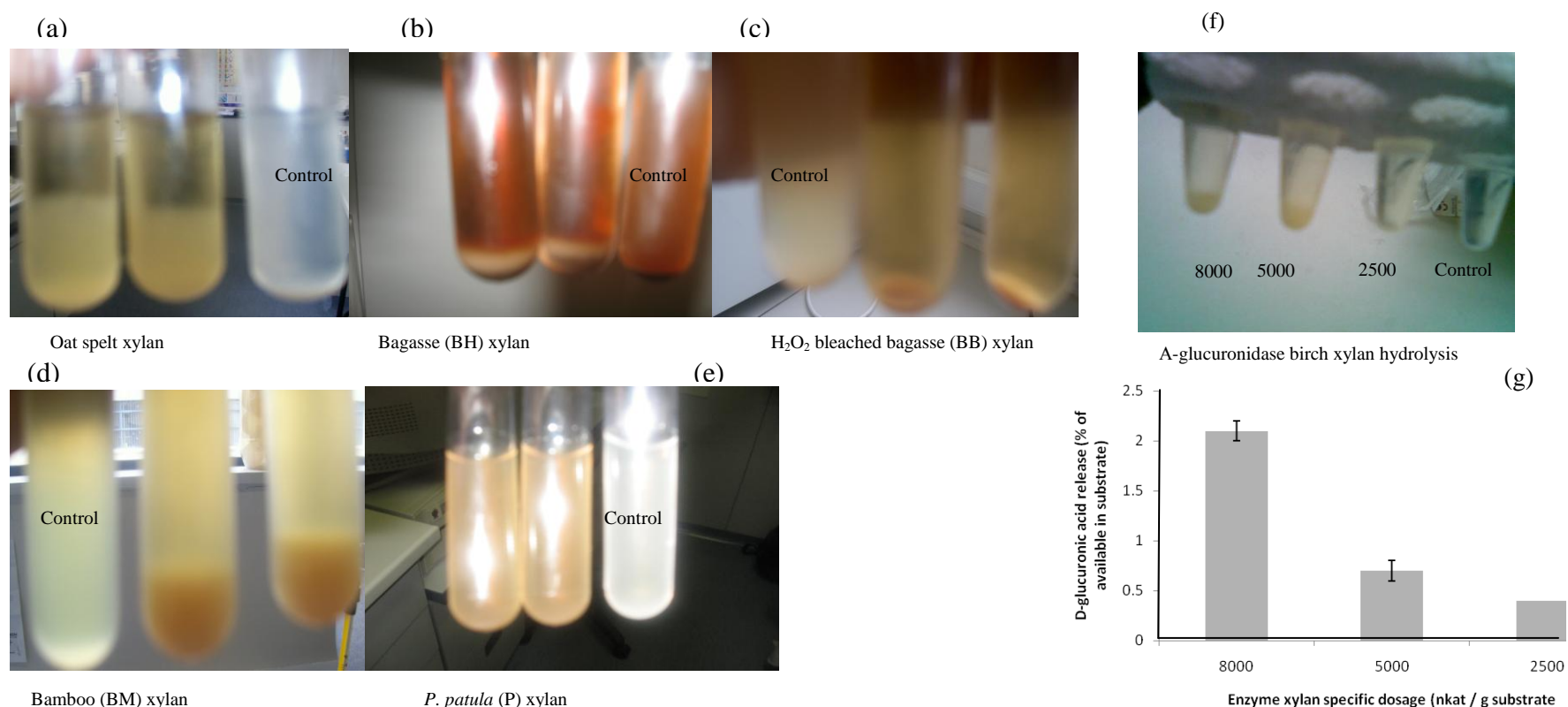


Figure 5.8: Formation of xylan hydrogel precipitates and aggregation after AbfB removal of arabinose side chains from xylan isolated from (a) oat spelt (b) bagasse (BH) (c) H₂O₂ bleached bagasse (BB), (d) bamboo (BM), and (e) *P. patula* (PP). The xylan was hydrolysed at 40°C, pH 5.0 for 16 h. (f) Xylan hydrogel precipitates aggregation after removal of MeGlcA side chains from birch xylan at 40°C, pH 4.8 for 24 h and (g) The degree of AguA removal of MeGlcA from birch xylan hydrolysed at 40°C, pH 4.8 for 24 h. The numbers 8000, 5000 and 2500 are xylan specific dosage (nkat g⁻¹ substrate) of purified α-D-glucuronidase.

5.3 DISCUSSION

5.3.1 Reducing solubility of xylans by enzymatic methods

The application of the recombinant α -L-arabinofuranosidase (AbfB) from *A. niger* that selectively removed arabinose side chains from oat spelt arabinoxylans and arabinoglucuronoxylans extracted from bagasse and bamboo feedstocks resulted into decreased solubility of the xylans. The reduction in the solubility of arabinoglucuronoxylans extracted from bagasse (Fig. 5.3b and 5.8b) and bamboo (Fig. 5.3d and 5.8d) was reflected by the increase in the viscosity and the precipitation upon selective removal of their arabinose side chains. Similar increase in viscosity and the precipitation of xylans were observed with selective removal of the arabinose side chains from the oat spelt xylan by the recombinant AbfB (Figs. 5.3a and 5.8a). However, the results showed that the selective removal of arabinose side chains from arabinoglucuronoxylan extracted from *P. patula* by the recombinant AbfB neither increased the viscosity (Fig. 5.3e) nor resulted into formation of xylan hydrogel precipitates (Fig. 5.8e).

The observed changes in viscosity and the precipitation of the oat spelt, bagasse and bamboo xylans could suggest that the recombinant AbfB selective removal of the arabinose side chains from these substrates created linear regions in the xylan main chain that resulted in increased attractive forces between and within the xylans. Kabel et al. (2007) suggested that the presence of a critical linear length of at least 15 xylose units on the main xylan chain to be the prerequisite for the formation and precipitation of the xylan hydrogels. Therefore, the unsubstituted regions of the xylans as a result of side chain removal are the driving force for increased viscosity and xylan hydrogel precipitation because the steric hindrance within and between the xylans is reduced, leading to precipitation of the xylan fractions (Ebringerová and Heinze, 2000). Kabel et al. (2007) also indicated that different xylan conformations arise from existence of varying degree of attractive forces within and between the xylan as a result of different degrees of xylan substitution and substitution pattern. Furthermore, Höije et al. (2008) reported that arabinoxylans precipitated into insoluble hydrogels when the degree of arabinose substitution, i.e. arabinose to xylose ratio, was lower than 0.3. In view of such precipitation conditions, the results in this study suggest that the selective removal of arabinose side groups from arabinoglucuronoxylans extracted

from *P. patula* by the recombinant AbfB did not create regions of unsubstituted xylose within the main xylan chain with required critical length for precipitation and the formation of hydrogels to occur. Therefore, the similarities in the viscosity changes and xylan precipitation due to the selective removal of the arabinose from arabinoglucuronoxylans extracted from bagasse and bamboo by the recombinant AbfB may be explained by the similarities in the degree and pattern of arabinose substitution to that of the oat spelt xylan (Chapter 3). For instance, the xylan extracted from bamboo had arabinose to xylose ratio of 1:7.6, which was similar to the arabinose to xylan ratio of oat spelt xylan (Table 5.1). On the other hand, the arabinose to xylose ratio of the *P. patula* xylan was 1:4 (Table 5.1) suggesting that the *P. patula* xylan was heavily substituted with arabinose side chains compared to the xylan extracted from bamboo.

The selective removal of the MeGlcA side chains did not result in formation of xylan hydrogel precipitates of the water soluble arabinoglucuronoxylans from bagasse, bamboo and *P. patula* as well as of the 4-*O*-methylglucuronoxylan extracted from *E. grandis* but of only the commercially obtained birch glucuronoxylan (Fig. 5.8f). The failure of the purified AguA to reduce the solubility of the arabinoglucuronoxylans extracted from bagasse, bamboo and *P. patula* and glucuronoxylan extracted from *E. grandis* upon the selective removal of the MeGlcA side chain, could similarly, be attributed to the differences in the degree of MeGlcA substitution and pattern and the presence of other side groups between the xylans. The chemical composition of the xylans extracted from bagasse, bamboo and *E. grandis* showed MeGlcA to xylose ratio ranging from 1:5.3- 1:1.75, which is higher than the MeGlcA to xylose ratio of almost 1:9 observed for the birch xylan. In addition, the birch xylans were deacetylated and substituted with only MeGlcA side chains (Chapter 3). In contrast, the chemical composition of the *E. grandis* glucuronoxylan showed evidence of acetylation despite alkaline hydrolysis and that of arabinoglucuronoxylans from bagasse, bamboo and *P. patula* showed high degree of arabinose side chains substitution (Chapter 3).

The purified AguA removed MeGlcA side groups to higher levels (50% of the available MeGlcA) that led to precipitation of the mainly from deacetylated glucuronoxylans compared to the corresponding acetylated glucuronoxylans (10% of

the available MeGlcA (Tenkanen and Siika-aho, 2000). However, it should be noted that the substrate specificity of the purified AguA reported by Tenkanen and Siika-aho (2000) is rather confusing because the purified AguA failed to increase the removal of MeGlcA upon removal of the acetyl groups by esterases, yet was able to release high amounts of MeGlcA from the water soluble deacetylated birch xylan. Therefore, the limited removal of the MeGlcA side chain by the AguA from the acetylated substrates cannot be attributed to the presence of the acetyl group. On the other hand, the observation that the purified AguA had high preference for the removal of MeGlcA from water soluble arabinoglucuronoxylans such as those extracted from bagasse (Fig. 5.1b) compared to birch glucuronoxylan and those extracted from *E. grandis* (Fig. 5.2) was in agreement with that of Tenkanen and Siika-aho (2000). In this study the purified AguA removed 50% more MeGlcA from arabinoglucuronoxylans (Fig 5.1b) compared to glucuronoxylans of birch and *E. grandis* (Fig. 5.2). The results of Tenkanen and Siika-aho (2000) showed that the purified AguA was capable of removing almost all (96%) MeGlcA side chains from arabinoglucuronoxylans compared to 50% degree of MeGlcA removal from deacetylated birch glucuronoxylan. Tenkanen and Siika-aho (2000) indicated that the action of the purified AguA was affected substantially by the degree and nature of substitution of the xylans. However, Tenkanen and Siika-aho (2000) neither reported on the changes in viscosity nor precipitation associated with the selective removal of the MeGlcA from the arabinoglucuronoxylans after hydrolysis with the purified AguA.

5.3.2 Role of recombinant AbfB and purified AguA in xylan processing

The formation of xylan hydrogels precipitates from water soluble xylan extracted from oat spelt, bagasse and bamboo upon selective hydrolysis by the recombinant AbfB and of birch xylan by the purified AguA is a clear indication that the recombinant AbfB and the purified AguA have potential application in production of high value speciality coatings and encapsulation matrices for use as speciality carriers or entrapments matrices of bioactive or immobilised biological compounds. These products have potential novel applications beyond the pulp and paper industries including biomedical engineering, pharmaceutical, food and cosmetic industries. This is because the xylans with reduced solubility are known to possess increased binding power that promote fibre to fibre bonding and improve the surface properties as

demonstrated with other types of xylans by Dahlman et al. (2003), Köhnke et al. (2008), Danielsson, (2007) and Kabel et al. (2007), Dahlman et al. (2003), Köhnke et al. (2008), Danielsson, (2007) and Kabel et al. (2007).

The ability to alter the viscosity of the xylans by the selective removal of the side chains is of special interest in the production of speciality thickening, antifoaming and emulsifying agents. The rheological properties of xylans are limited but so far have been studied in relation to bread making (Rattan et al., 1994). Ebringerová et al. (2005) indicated that the high intrinsic viscosity is an indication of the emulsifying effect of the xylans. Therefore, the differences in the change of viscosity of the xylan that occurred with the selective removal of the arabinose side chains by the AbfB from bamboo, bagasse and oat spelt xylans and that of xylan extracted from *P. patula* could form the basis for choosing the appropriate xylan sources for special end uses as demonstrated in the improvement bread quality by Biliaderis et al. (1995). The results in Figures 5.3a, 5.3b and 5.3d showed that the oat spelt, bagasse and bamboo xylans respectively, selectively hydrolysed by the recombinant AbfB had higher apparent viscosity relative to the corresponding untreated xylans. However, the apparent viscosity of both the enzyme treated and untreated xylans reduced with increased shear rate. Ebringerová et al. (2005) associated such effect to the thixotropic and shear thinning behaviour of less substituted xylan when in aqueous solutions compared to only the shear thinning property of highly substituted xylans.

5.3.3 Synergetic effect of recombinant AbfB and purified AguA on removal of side groups and precipitation of polymeric xylans

Both the recombinant AbfB and the purified AguA present potential biological tools for functionalisation of xylans that could lead to production of different novel products. However, the activity of the recombinant AbfB and purified AguA but in this study depended on the structural properties of the xylans, thus the type, degree and pattern of side chain substitution on the main xylan chain. Therefore, the synergetic effect of the recombinant AbfB and AguA and other side chain removing enzymes may be necessary to enhance the change in viscosity and precipitation of the arabinoglucuronoxylans extracted from bagasse, bamboo and *P. patula*. The results showed that there was a more positive synergetic effect between the recombinant

AbfB and purified AguA for the removal of arabinose side chains from the arabinoglucuronoxylans extracted from bagasse, bamboo and *P. patula* than the removal of MeGlcA side chains from these xylans (Figs. 5.1a and 5.1b). For example, the simultaneous application of recombinant AbfB and purified AguA enzymes (i.e. the recombinant AbfB and purified AguA enzymes were added at the same time in the reaction mixture) on xylan extracted from bagasse by the Hoiye method (Chapter 3) increased the arabinose removal by 21% (Fig. 5.1a) whereas, the removal of MeGlcA increased by only 13% (Fig. 5.1b). Furthermore, the hydrolysis by combined application of the recombinant AbfB and the purified AguA enzymes of xylan extracted from bamboo by the Hoiye method (Chapter 3) increased the arabinose removal by 33% (Fig. 5.1b) but reduced the removal of the MeGlcA by the purified AguA by 13% (Fig. 5.1b).

The limited or lack of increase in the degree of removal of MeGlcA side chains by the purified AguA from the bagasse, bamboo and *P. patula* arabinoglucuronoxylans during combined application with the recombinant AbfB could probably be associated with the problems of restricted access or lack of recognition of the glucuronic side chains on the main xylan chain due to conformational changes caused by the removal of the arabinose side chains. The lack of synergistic effect between the AbfB and purified AguA on the removal of glucuronic side chains from arabinoglucuronoxylans was earlier observed by Tenkanen and Siika-aho (2000) during hydrolysis of xylans extracted from spruce wood and spruce pulp whereby, the increase in the removal of arabinose side groups was possible but not the increase in the removal of MeGlcA. Furthermore, Tenkanen and Siika-aho (2000) also observed reduced increase in the removal of MeGlcA from acetylated birch xylan by the AguA when working in concert with acetyl xylan esterases that removed the acetyl groups in acetylated birch xylan. It appears that the action of the AguA is not only hindered by the presence of the other side chains (Tenkanen and Siika-aho, 2000) but also by the conformation changes in structure of the substrate as a result of the action of other side chain removing enzymes. Such changes could affect the AguA's substrate accessibility and recognition. It is reported that the molecular sizes of the AguA, which is as high as 180 kDa (Castanares et al. 1995, Tenkanen and Siika-aho, 2000; De Wet and Prior, 2004) could be restrictive to access its substrates. In addition, most α -D-glucuronidases are known to possess complex substrate recognition systems, which

could be affected by changes in xylan chain length, the structural arrangement and the position of the MeGlcA they attack on the xylan main chain and the presence or absence of other functional groups (Siika-aho et al., 1994; Tenkanen and Siika-aho, 2000; Nagy et al., 2003; Biely, 2003; De Wet and Prior, 2004). However, the AguA with polymeric xylan substrate specificity hydrolyzes the α -1, 2-glycosidic bond between the MeGlcA and single xylose moiety located at the non-reducing end of xylooligosaccharides (Siika-aho et al., 1994; Biely, 2003). Therefore, the lack of reduction of water solubility of the arabinoglucuronoxylans could be attributed to the change in substrate specificity on heterosubstituted xylans. Relatively, the recombinant AbfB would be a preferred enzyme for the precipitation of the arabinoglucuronoxylans, probably because of its inherent broad but restricted substrate specificity.

5.3.4 Conditions for reducing solubility of water soluble polymeric xylans

Modification of solubility properties with respect to viscosity and precipitation during hydrolysis of xylans by the recombinant AbfB and purified AguA was influenced by the enzyme xylan specific dosage, hydrolysis time and temperature. Therefore, customised removal of the side chains and manipulation of conditions for selective hydrolysis of the xylans by the recombinant AbfB and purified AguA could possibly enable the xylans to possess structures that would enhance precipitation and formation of hydrogels to suit special end uses. This idea is based on the results that showed that the viscosity of xylans extracted from bamboo increased by increasing the recombinant AbfB xylan specific dosage from 180.0 to 540.0 nkat g⁻¹_{substrate} (Fig. 5.4a). Similarly, the viscosity of the oat spelt xylan decreased by increasing the temperature from 40 to 60°C (Fig. 5.4b).

The differences in the viscosity changes of the polymeric xylan treated with the recombinant AbfB under the given hydrolysis condition could be attributed to the increased degree of removal of the arabinose side groups from the xylans at 40°C and at higher recombinant AbfB xylan specific dosage (720.0 nkat g⁻¹_{substrate}) compared to the release of arabinose at 60°C and at lower AbfB xylan specific dosage (180.0 nkat g⁻¹_{substrate}). However, the hydrolysis time and arabinose removal from oat spelt xylan by the recombinant AbfB had a non-linear relationship, which yielded a significant negative correlation (*r*) of -0.544) at *p* < 0.05 (Fig. 5.5) with viscosity. Therefore,

factors that influence degree of side chain removal would also impact on the viscosity properties of the xylans.

However, the negative relationship between viscosity and degree of arabinose removal suggests that although the viscosity of the oat spelt xylan increased with application of the recombinant AbfB (Fig. 5.3 a), such viscosity is not sustained with time, hence the observed decreasing trend displayed in particular with increased hydrolysis time (Fig. 5.7a-c). The decrease in viscosity of the oat spelt xylan with time could be as a result of phase separation due to aggregation of the xylan hydrogel precipitates. Such behaviour could possibly be explained by the thixotropic and shear thinning behaviour that prevail with less substituted xylans as reported by Ebringerová et al. (2005). The surface plots for viscosity fitted with the second order polynomial as a function of time and temperature and AbfB xylan specific dosage ($\text{nkat g}^{-1}_{\text{substrate}}$) showed that the viscosity decreased to the lowest value of 2.03 mPa.s (measured at shear rate of 10.3 s^{-1}) at hydrolysis time of 9 h and temperature of 45.8°C with recombinant AbfB xylan specific dosage of $\approx 400.0 \text{ nkat g}^{-1}_{\text{substrate}}$ (Fig. 5.7a-c). However, the results showed that beyond 10 h an increase in viscosity of up to $\geq 4 \text{ mPa.s}$ prevailed (Figs. 5.7a and 5.7b). Therefore, depending on the hydrolysis time and degree of arabinose removal from the oat spelt xylan at which the viscosity is measured, an increasing or decreasing trend of the change in viscosity could prevail.

The removal of arabinose side chains increased with increase in recombinant AbfB enzyme xylan specific dosage during hydrolysis of oat spelt xylan (Fig. 5.6). However, the degree of precipitation of the oat spelt xylan did not increase any further with increase in the recombinant AbfB xylan specific dosage after release of 10% of the available arabinose (Fig. 5.6). The levelling off of the precipitation of the oat spelt xylan with increased arabinose removal was probably a consequence of the AbfB continued removal of the arabinose from xylan that had already precipitated from the solution (Fig. 5.8a) rather than from unmodified xylan still present in the solution. Similarly, the degree of purified AguA removal of the MeGlcA from birch xylan increased 5 times by increasing the purified AguA xylan specific dosage from 2500 to 8000 $\text{nkat g}^{-1}_{\text{substrate}}$. In addition, the precipitated birch xylan hydrogels were visible in the reaction mixture in which birch xylans were treated with purified AguA at xylan specific dosage of 2500 $\text{nkat g}^{-1}_{\text{substrate}}$ (Fig. 5.8g). However, the volume of

the birch xylan hydrogel precipitates was not significantly different for that of the birch xylans treated with the purified AguA at xylan specific dosage 5000 and 8000 nkat g⁻¹_{substrate} (Fig. 5.8g). Notably, the maximum degree of removal of the MeGlcA of 2.5% of the available MeGlcA from the birch xylans was reached with purified AguA xylan specific dosage of 5000 nkat g⁻¹_{substrate} (Fig. 5.8f). The release of MeGlcA side chains from the birch xylan by the AguA obtained in this study constituted 5% of MeGlcA side chain removal reported for birch xylan by Puls (1992) and Tenkanen and Siika-aho (2000). The large difference between the results of this study those results may be attributed to the differences in the hydrolysis conditions and possibly the methods for quantifying the MeGlcA, which used glucuronic acid as the standard sugar.

5.4 CONCLUSION

Application of the recombinant α -L-Arabinofuranosidase (AbfB) from *A. niger* and purified α -D-glucuronidase (AguA) from *S. commune* offer a potential novel technique for decreasing solubility and formation of insoluble hydrogels from bamboo, *E. grandis* and bagasse polymeric xylans. Such technique is required in lignocellulosic processing for production of speciality coatings, additives and encapsulation matrix. The recombinant AbfB and purified AguA both individually and in combination, selectively removed arabinose and MeGlcA side chains respectively, from polymeric xylans pre-extracted from wood and grass sources that led to varying degrees of viscosity change and precipitation. The combined application of the recombinant AbfB and purified AguA increased the removal of arabinose side chains from arabinoglucuronoxylans but not the removal of MeGlcA side chains by the purified AguA. The reduction in the solubility state of the xylans was attributed to both the degree of side chain removal and change in substitution pattern that was dependant on the structural properties of the xylan, enzyme xylan specific dosage, hydrolysis time and temperature. Therefore, optimisation of the enzyme xylan specific dosage, hydrolysis time and temperature during hydrolysis of xylans by the recombinant AbfB and AguA for specific xylan is required for customised removal of the side chains for precipitation and hydrogel formation to ensure efficient use of both the enzymes and the xylan.

Chapter 6: Optimal conditions for selective removal of side chains and precipitation of polymeric xylans using enzymatic methods

ABSTRACT

The optimal values of hydrolysis time, enzyme dosage and temperature for the selective removal of arabinose side chains by recombinant α -L-arabinofuranosidase (AbfB) and of MeGlcA side chains by purified α -D-glucuronidase (AguA) from oat spelt and birch xylans respectively and the subsequent precipitation effect were determined by the Box-Benhken response surface method (RSM). The experimental region covered the xylan specific dosage for recombinant AbfB between 18.0 and 540.0 $\text{ngatg}^{-1}_{\text{substrate}}$ and for purified AguA between 2000 and 18 000 $\text{ngatg}^{-1}_{\text{substrate}}$ at temperatures between 30 and 50°C and hydrolysis time between 1 and 16 h. The optimal values for hydrolysis time, temperature and xylan specific dosage were estimated to be 14-16 h, 38-45°C and 607.0 $\text{ngatg}^{-1}_{\text{substrate}}$ respectively, for maximum removal of 43% of the available arabinose in oat spelt xylan by the recombinant AbfB. Whereas, the optimal values for hydrolysis time, temperature and xylan specific dosage for the selective removal of MeGlcA by the purified AguA were estimated at 11 h, 38°C and 18 000 $\text{ngatg}^{-1}_{\text{substrate}}$ respectively, for maximum removal of 0.5% MeGlcA from the birch xylan. The enzyme xylan specific dosage, hydrolysis time and temperature showed significant effect ($p < 0.05$) on both recombinant AbfB arabinose removal from oat spelt xylan and purified AguA MeGlcA removal from birch xylan, but only the interactions between the hydrolysis time and temperature were significant ($p < 0.05$) on recombinant AbfB arabinose removal but not on purified AguA removal of MeGlcA from birch xylan. The optimal values of the hydrolysis parameters for AbfB removal of the arabinose from oat spelt xylan and AguA removal of MeGlcA from birch xylan could be predicted using quadratic models that fitted the response surface plots with regression coefficient of > 0.9 .

6.0 INTRODUCTION

Methods for controlled removal of the side groups from xylan in order to enhance the xylan gelling and binding properties to be similar to those of high molecular weight polysaccharides are lacking. The complexity of the degree of substitution and substitution pattern of the xylans is particularly a limiting factor to customised reduction of its solubility (Ebringerová and Heinze, 2000; Lindblad et al., 2001). The variations in the type and degree of side group substitution on xylan main chain was evident in the structures of xylans extracted from South African feedstocks (Chapter 3), which influenced their solubility and response to selective hydrolysis (Chapter 5). The xylans are made of β -1, 4-linked xylopyranosyl unit backbone to which either α -L-arabinofuranoside (arabinose), or 4-*O*-methyl-D-glucuronic acid (MeGlcA) or both side groups are attached to varying degrees depending on the lignocellulosic source (Timell, 1967; Wilkie, 1979). The xylans extracted from hardwood may be acetylated while those from grasses might have arabinofuranosyl residues that are esterified with ferulic or *p*-coumaric acid groups (Timell, 1967; Wilkie, 1979) (Chapter 3). The presence of these side groups causes steric hindrance, which prevents hydrogen bonding within and between the xylan chains (Viikari et al., 1993; Izydorczyk and Billaderis, 1995; Ebringerová and Heinze, 2000). Consequently, the substituted xylans is rendered highly soluble in water with less adhesive power and ability to form hydrogels or biofilms similar to starch and cellulose.

The recombinant α -L-arabinofuranosidase (AbfB) from *Aspergillus niger* belonging to glycoside hydrolases family (GH) 54 (Chapter 4) and the purified α -D-glucuronidase (AguA) (EC 3.2.1.139/131) from *Schizophyllum commune* (Tenkanen and Siika-aho, 2000) have a critical role in diversifying the functional properties of water soluble xylans. The recombinant AbfB and purified AguA selectively removed arabinose and MeGlcA side chains respectively from polymeric xylans derived from grass and wood sources, which resulted into different degrees of viscosity change and hydrogel formation and precipitation (Chapter 5). Kabel et al. (2007) showed that side group removing enzymes could be used to obtain xylans with varying degree of substitution and substitution patterns of the side groups that possessed varying levels of binding power towards cellulosic materials. Similarly, Höije et al. (2008) used side chain removing enzymes to obtain specific degree of substitution that resulted in enhanced film forming properties of arabinoxylans. The findings from these studies

imply that the side group removing enzymes present a flexible alternative method for reducing the water solubility of the xylans.

The important parameters that influence the degree of side chains removal from xylans were identified as time, temperature, enzyme dosage (Chapter 5). The removal of side chains at optimal conditions would result in efficient utilization of both the xylans and the enzyme, which are scarce resources of high economic value. So far, optimal conditions for application of the recombinant AbfB and the purified AguA, individually and in combination for selective removal of arabinose and MeGlcA side chains respectively, have not been identified because in most studies, the enzymatic hydrolysis parameters were often not optimized simultaneously. In a study by Tenkanen and Siika-aho (2000) the removal of MeGlcA from birch xylan by purified AguA stagnated despite increasing either hydrolysis time or enzyme dosage. Similar observation was made by Margolles and De Los Reyes-Gavilán (2003) on the removal of arabinose side chains from wheat arabinoxylan by α -L-arabinofuranosidase of *Bifidobacterium longum* B667.

The application of the enzymes for selective hydrolysis of xylans at industrial level would require identification of optimal conditions. Therefore, the purpose of the study was to determine the level of influence and optimal values of the xylan concentration, enzyme dosage, hydrolysis time and temperature on the degree of selective removal of side chains and precipitation of model polymeric xylans substituted with arabinose side chains (oat spelt) and MeGlcA side chains (birch xylan) by the recombinant AbfB and purified AguA respectively using the response surface method (RSM).

6.1 MATERIALS AND METHODS

6.1.1 Materials

Oat spelt xylan (Sigma, X-0627) with a sugar composition of 10:15:75 (arabinose:glucose:xylose) and birch xylan (Roth, 7500) with a sugar composition of 8.3:1.4:89.3 (MeGlcA:glucose:xylose) as reported by Kormelink and Voragen (1993) were made into 1% (w/v) solutions according to protocols described by De Wet et al. (2008). The solutions were made in bulk and stored in vials at -20°C.

Crude α -L- arabinofuranosidase (AbfB) produced by recombinant *A. niger* D15 with volumetric activity of 18.0 nkat mL⁻¹ against *para*-nitrophenyl- α -L- arabinofuranoside (*p*NPA) was produced in house (Chapter 4). In addition, α -D- glucuronidase (AguA) isolated and purified from wild type *S. commune* (VTT-D-88362- ATCC 38548) with specific activity of 300 nkat mg⁻¹ was generously donated by Prof. Matti Siika-aho of VTT Biotechnology Institute, Finland. The enzyme aliquots were stored at 4°C for the entire duration of the study. L (+) - arabinose (Sigma, A3256) and D-MeGlcA (Sigma, G5269D) were used as standard sugars.

6.1.2 Effect of hydrolysis parameters on enzymatic removal of side chains and precipitation of polymeric xylans

Oat spelt xylan was incubated with AbfB in 400 μ L reaction mixtures containing 200 μ L of the substrate, with AbfB xylan specific dosage ranging from 44.0-140.0 nkat g⁻¹_{substrate} in 0.05 M citrate buffer pH 5. The reactions were performed in a water bath set at 40°C for durations of 2, 4, 8 and 16 h. The effect of temperature was assessed in 5 mL reaction mixtures into which 2.5 mL of substrate was added and incubated with AbfB at xylan specific dosage of 180.0 and 720.0 nkat g⁻¹_{substrate} at 40 and 60°C for 4 and 16 h. The reactions were stopped by either boiling the reaction mixture for 10 min or by immediately placing the samples on ice. The xylan hydrolysates were analysed for arabinose release. Noteworthy, this experiment was performed only with AbfB because AguA was available in limited quantities.

6.1.3 Determining optimal conditions for enzymatic hydrolysis of polymeric xylans

Optimal values for time, temperature and enzyme dosage for the AbfB removal of arabinose from oat spelt xylan and the AguA removal of MeGlcA from birch xylan were determined in a three factor Box-Behnken response surface statistical design with 3 central points, making a total of 15 runs (Statistica 7.0 software programme, StatSoft, Inc., 1984-2005). The hydrolysis parameters were each tested at two levels and middle point with the highest, middle and lowest levels denoted as 1, 0 and -1 respectively. Temperature was tested at 30 and 50°C, time at 1 and 16 h and enzyme dosage for recombinant AbfB at 180.0 and 540.0 nkat g⁻¹_{substrate}. The temperature and hydrolysis time set points for MeGlcA side chain removal by the purified AguA were the same as those of recombinant AbfB removal of arabinose side chains but the xylan specific purified AguA dosage was at 2.0 and 18.0 µkatg⁻¹_{substrate}. The central points for temperature and time were 40°C and 8.5 h, while for AbfB and AguA xylan specific dosages were 0.4 and 11.0 µkat g⁻¹_{substrate} respectively. The full experimental set up with runs presented in random order for arabinose removal from oat spelt xylan and MeGlcA from birch xylan are presented in Table 6.1 and 6.2 respectively. The treatment runs were conducted in duplicates. The variables were coded according to equation 6.1.

$$x_i = \left(\frac{X_i - \bar{X}_i}{\Delta X_i} \right) \quad (6.1)$$

Where:

x_i = coded value for variables

X_i = natural value

ΔX_i = scaling factor (half the range of the independent variables, which constituted time, temperature and enzyme xylan specific dosage)

The effects of xylan concentration and enzyme activity on the removal of arabinose side chains from oat spelt xylan by the recombinant AbfB as individual parameters were assessed in a two factor cube plus star central composite statistical design experiment consisting 10 runs. The experiments were performed in a reaction mixture of 8 mL, which contained oat spelt xylan solution treated with varying enzyme

dosages (nkat g⁻¹_{substrate}) according to the experimental design in Table 6.3. The reaction was done in a water bath set at 40°C for 16 h. The hydrolysed samples were analysed for arabinose concentration to determine degree of arabinose removal. The response surface plots were fitted with a second order polynomial, which included both linear and quadratic interactions as follows:

$$Z = \beta_0 + \beta_1 X_1 + \beta_{11} X_1^2 + \beta_2 X_2 + \beta_{22} X_2^2 + \beta_3 X_3 + \beta_{33} X_3^2 + \beta_{112} X_1^2 X_2 + \beta_{113} X_1^2 X_3 + \beta_{122} X_1 X_2^2 + \beta_{133} X_1 X_3^2 + \beta_{223} X_2^2 X_3 + \beta_{233} X_2 X_3^2 + \beta_{1122} X_1^2 X_2^2 + \beta_{1133} X_1^2 X_3^2 + \beta_{2233} X_2^2 X_3^2 + \varepsilon \quad (6.2)$$

Where:

Z = Response (degree of side chain removal).

$\beta_0, \beta_1, \dots, \beta_n, \dots$ = Linear regression coefficients.

$\beta_{11}, \dots, \beta_{nn}, \dots$ = Quadratic regression coefficients.

X_1, X_2, X_3 = Hydrolysis time, temperature and enzyme xylan specific dosage or xylan and enzyme loading respectively.

ε = Error

6.1.5 Sugar analysis

Arabinose and glucuronic side chain sugars were analysed using high performance anion exchange chromatography coupled with pulsed amperometric detector (HPAEC-PAD (Dionex)), which was equipped with a gradient pump GP 50, a CarbopacTM PA 10 (4 mm x 250 mm) column and electrochemical detector (ED40). The eluents were 250 mM NaOH and Milli-Q-water in a ratio of 1.5:98.5 at a flow rate of 1 mL min⁻¹. Notably, sodium acetate (1M NaOAc) was used only when analysing MeGlcA side chain sugars (Chapter 5). The sugar samples were filtered on 0.22 µm pore size filters before analysis on HPAEC-PAD (Dionex). The quantity of the sugars was determined from standard plots of analytical grade L (+) arabinose and D-MeGlcA standard sugars

6.1.6 Statistical analysis

Statistical analyses for the study included regression analysis and analysis of variance (ANOVA). In addition, Pareto Chart plots were used to present size and significance of effects and the desirability and profiling function was used to locate optimal set point values for the hydrolysis parameters.

Table 6.1: Box-Behnken statistical design for determining effects of hydrolysis parameters on removal of arabinose side chains from oat spelt xylan by recombinant AbfB.

Treatment run	Coded variables			Natural variables		
Random order	X1	X2	X3	Time (h)	Temperature (°C)	Enzyme dose (nkat/g)
9	0	-1	-1	8.5	30	180.0
3	-1	1	0	1	50	360.0
10	0	1	-1	8.5	50	180.0
6	1	0	-1	16	40	360.0
11	0	-1	1	8.5	30	180.0
4	1	1	0	16	50	540.0
7	-1	0	1	1	40	360.0
14	0	0	0	8.5	40	540.0
12	0	1	1	8.5	50	360.0
15	0	0	0	8.5	40	540.0
5	-1	0	-1	1	40	360.0
2	1	-1	0	16	30	180.0
13	0	0	0	8.5	40	360.0
8	1	0	1	16	40	360.0
1	-1	-1	0	1	30	540.0

Design summary: 3 factor Box-Behnken design, number of factors independent variables: 3, number of runs (cases, experiments): 15 and number of blocks: 1

Table 6.2: Box-Behnken experimental set up for selective removal of MeGlcA side chains from birch xylan by purified AguA

Treatment (Random order)	Run	Coded variables			Natural variables		
		X1	X2	X3	Enzyme dose [μ kat Substrate (g^{-1})]	Time (h)	Temperature ($^{\circ}\text{C}$)
6		1	0	-1	18.0	8.5	30
15		0	0	0	10.0	8.5	40
1		-1	-1	0	2.0	1.0	40
14		0	0	0	10.0	8.5	40
8		1	0	1	18.0	8.5	50
5		-1	0	-1	2.0	8.5	30
2		1	-1	0	18.0	1.0	40
13		0	0	0	10.0	8.5	40
11		0	-1	1	10.0	1.0	50
9		0	-1	-1	10.0	1.0	30
3		-1	1	0	2.0	16.0	40
10		0	1	-1	10.0	16.0	30
12		0	1	1	10.0	16.0	50
4		1	1	0	18.0	16.0	40
7		-1	0	1	2.0	8.5	50

***Design summary:** 3 factor Box-Behnken design, number of factors independent variables: 3, number of runs (cases, experiments): 15 and number of blocks: 1

Table 6.3: Central composite design for assessing effect of oat spelt xylan concentration and enzyme activity on selective removal of arabinose side chain from oat spelt xylan by the recombinant AbfB.

Treatment run	Coded variables		Natural variables	
Random order	A	B	xylan concentration ($\mu\text{g mL}^{-1}$)	*Enzyme dosage (nkat mL^{-1})
1	-1.00000	-1.00000	1562.50	9.0
6	1.41421	0.00000	7220.30	22.5
10 (C)	0.00000	0.00000	3906.30	22.5
4	1.00000	1.00000	6250.00	36.0
3	1.00000	-1.00000	6250.00	9.00
8	0.00000	1.41421	3906.30	41.0
5	-1.41421	0.00000	592.20	22.5
7	0.00000	-1.41421	3906.30	3.4
9 (C)	0.00000	0.00000	3906.30	22.5
2	-1.00000	1.00000	1562.50	36.0

Design summary: 2** (2) central composite, nc = 4, ns = 4, n0 =2, Runs=10 *values rounded off to significant numbers, C = central point run.

6.2 RESULTS

6.2.1 Effect of hydrolysis parameters on side chain removal and precipitation of polymeric xylans

The removal of the arabinose side chains from oat spelt xylan by the recombinant AbfB increased with increase in hydrolysis time and enzyme dosage (Fig. 6.1a). A maximum of 27% of the available arabinose was liberated from oat spelt xylan treated with recombinant AbfB for 8 h at xylan specific dosage level of $1\,400.0\text{ nkatg}^{-1}_{\text{substrate}}$ (Fig. 6. 2a). An equal amount of arabinose was released by treating the oat spelt xylan with the recombinant AbfB at xylan specific dosage of about $700.0\text{ nkatg}^{-1}_{\text{substrate}}$ for 16 h (Fig. 6.1a). However, the change in the degree of arabinose release from oat spelt xylan by the recombinant AbfB at hydrolysis time of $\geq 8\text{h}$ and recombinant AbfB xylan specific dosage level of $\geq 350.0\text{ nkat g}^{-1}$ was not significant ($p < 0.05$) (Fig. 6.1a). Irrespective of the hydrolysis time, the arabinose was not detectable from the AbfB hydrolysis of the oat spelt xylan at 60°C at a xylan specific dosage of $180.0\text{ nkatg}^{-1}_{\text{substrate}}$, (Fig. 6.1b). However, increasing the AbfB xylan specific dosage from 180.0 to $720.0\text{ nkatg}^{-1}_{\text{substrate}}$ at 60°C resulted in release of about 1.1% of the available arabinose from in the oat spelt xylan. On the other hand, the oat spelt xylan treated with AbfB at dosage level of $180.0\text{ nkatg}^{-1}_{\text{substrate}}$ at 40°C for 4h, released about 4% of the available arabinose (Fig. 6.1b) whereas, increasing the AbfB xylan specific dosage level to $720.0\text{ nkatg}^{-1}_{\text{substrate}}$ at 40°C yielded 14% of the available arabinose (Fig. 6.1b). The recombinant AbfB removal of the arabinose side chains caused precipitation of the oat spelt xylan solution leading to formation of hydrogel aggregates. The precipitation was visible after treatment with AbfB xylan specific dosage of $720.0\text{ nkatg}^{-1}_{\text{substrate}}$ at 40°C but not at 60°C (Fig. 6.1c).

6.2.2 Determination of optimal conditions for removal of arabinose and MeGlcA side chains

The response surface plots for the removal of arabinose from oat spelt xylan as a function of temperature, time and AbfB xylan specific dosage reflected both linear and quadratic relationships (Figs. 6.2a (i-iii)). A maximum of $12\text{ mg g}^{-1}_{\text{substrate}}$ of arabinose (12% of available arabinose) was liberated from oat spelt xylan for hydrolysis times ranging from 10.8 to 18 h with AbfB xylan specific dosages ranging

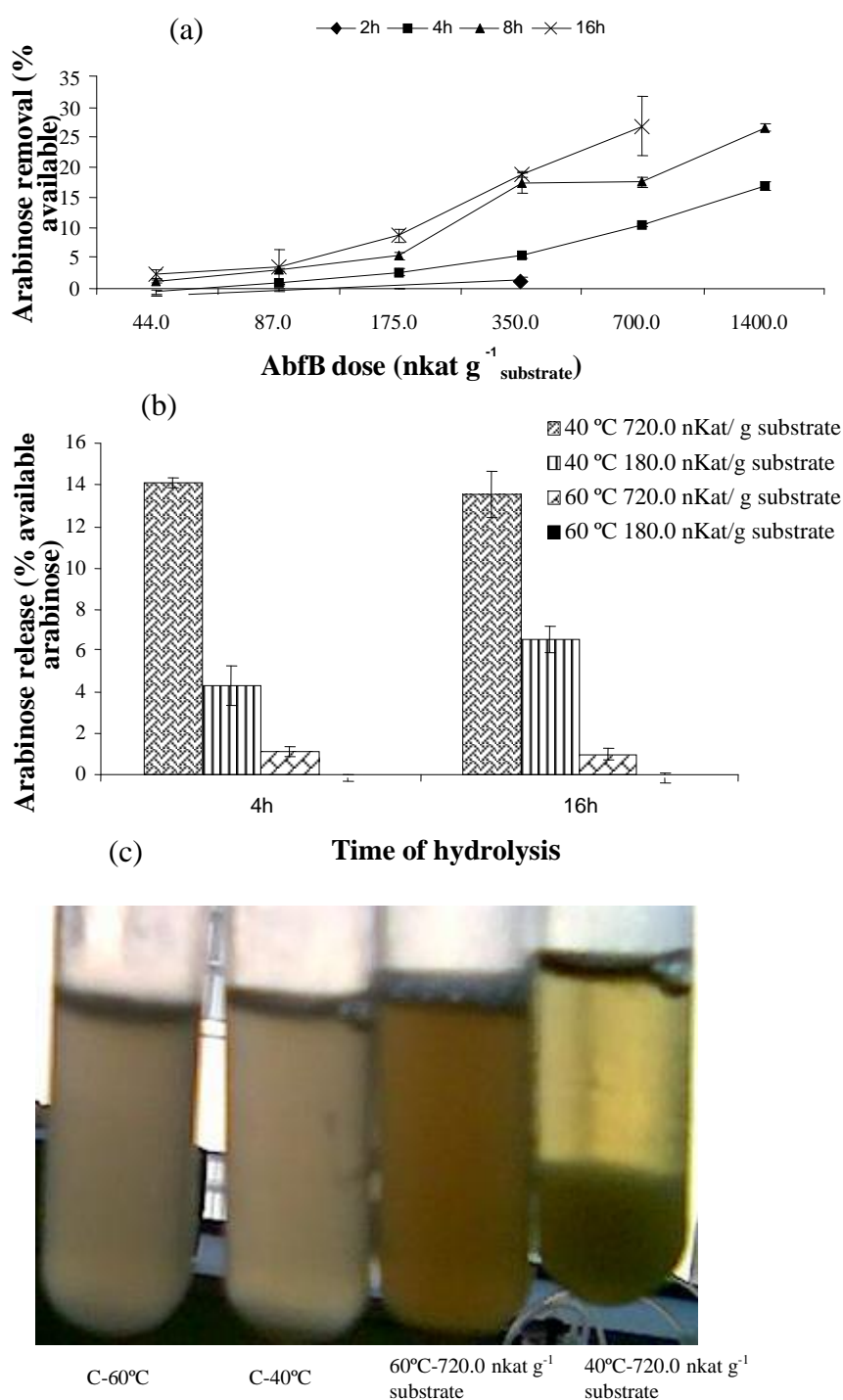


Figure 6.1: (a) Arabinose removal from oat spelt xylan by recombinant α -L-arabinofuranosidase (AbfB) at varying xylan specific dosage (nkat g⁻¹ substrate) and hydrolysis time [h]. The selective hydrolysis was performed at 40°C, and pH 5, (b) Effect of hydrolysis time (h) and temperature (°C) on selective arabinose removal from oat spelt xylan and (c) insolubilization and precipitation of oat spelt xylan by the recombinant AbfB at varying xylan specific dosages between 180 and 720.0 nkat g⁻¹ for 16 h at pH 5.0. Note: in (c) the C-40 /60 °C indicates control sample. Incomplete line graphs in part (a) were due to loss of samples during analysis.

from 427.0 to 725.0 nkat g⁻¹_{substrate} and at temperatures ranging from 35.9 to 44.5°C (Fig. 6.2a (i-iii)). Similarly, the response surface plots for removal of MeGlcA from birch xylan by AguA reflected both linear and quadratic relationship as a function of hydrolysis time, temperature and AguA xylan specific dosage (Figs. 6.2b (i-iii)). A maximum of 360 µg g⁻¹_{substrate} of MeGlcA (0.43% of available MeGlcA) was removed from birch xylan by AguA with xylan specific dosage between 16500 and 18000 nkat g⁻¹_{substrate} when the AguA hydrolysis was performed for durations between 8 and 10.2 h at temperatures between 33.5 and 42°C (Figs. 6.2b (i-iii)). The desirability contour plots showed optimal set points for AbfB removal of arabinose from oat spelt xylan to be 14 h, temperature of 40.5°C and enzyme dosage level at 550.0 nkatg⁻¹_{substrate} (Fig. 6.3a (i-iii)). Whereas, the optimal set points for AguA removal of MeGlcA were 11.0 h for time, temperature of 38°C and enzyme dosage level of 18000 nkat substrate (g)⁻¹ (Fig. 6.3b (i-iii)). The regression analysis on the data from the experiments of AbfB removal of arabinose as a function of time, temperature and enzyme dose provided regression coefficients (R²) of 0.99 (adjusted R² = 0.97) of the variables in the second-order polynomial model fitted to the response surface plots that included interactions (Table 6.4). The AguA removal of MeGlcA gave R² of 0.99 (R² adjusted = 0.94) (Table 6.5).

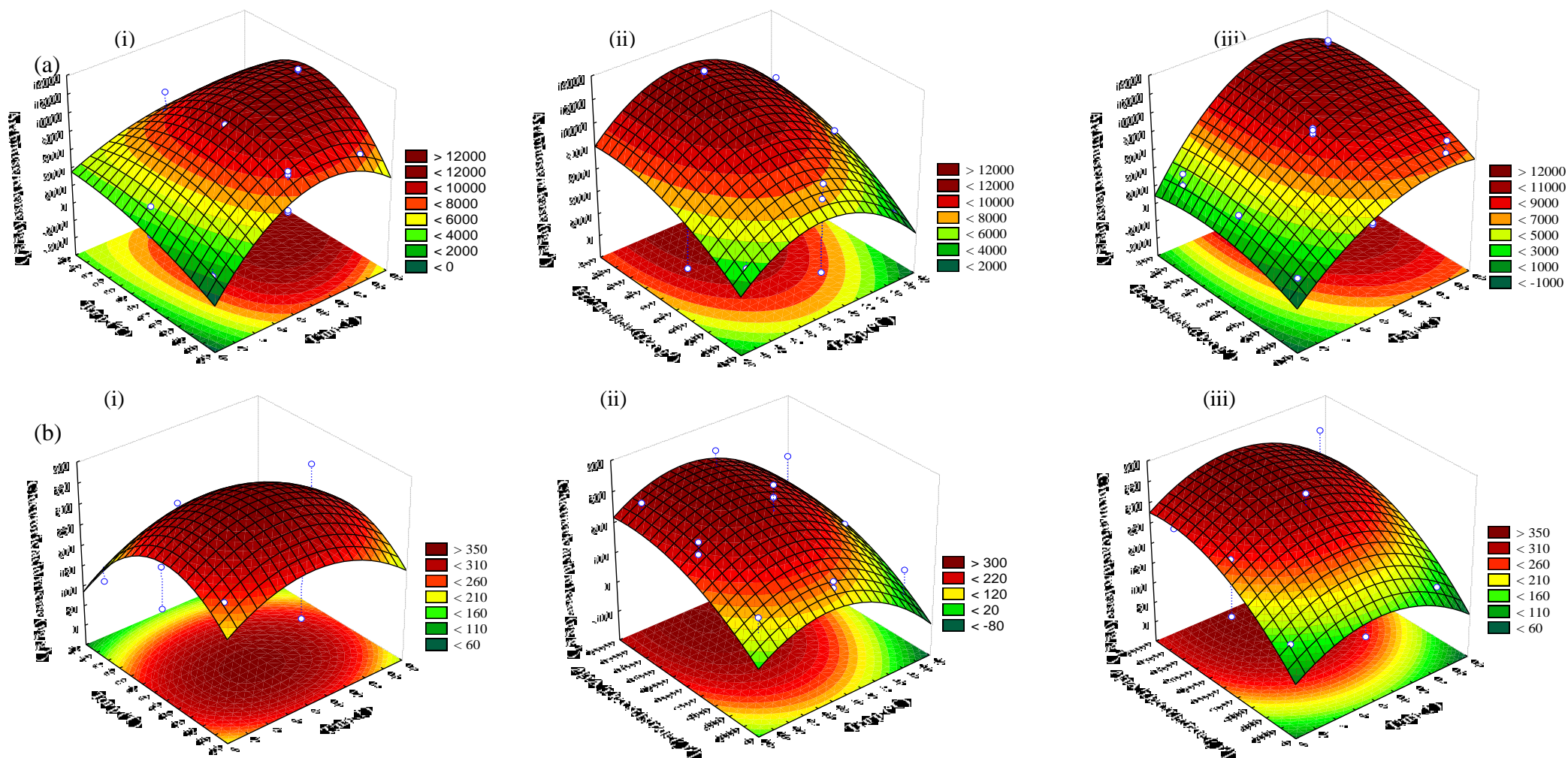


Figure 6.2: Response surface plots for (a) arabinose removal and (b) MeGlcA removal as a function of [i] time (h) and temperature ($^{\circ}\text{C}$), [ii] temperature ($^{\circ}\text{C}$) and enzyme dose ($\text{nkatg}^{-1}_{\text{substrate}}$) and [iii] time (h) and AbfB xylan specific dosage ($\text{nkatg}^{-1}_{\text{substrate}}$). The AbfB arabinose removal fixed conditions were (a) [i] AbfB dosage = $427.0 \text{ nkatg}^{-1}_{\text{substrate}}$, (a) [ii] time 10.8 h and a [iii] temperature 35.9°C . The AguA MeGlcA removal fixed conditions were (b) [i] AguA dosage = $16500 \text{ nkatg}^{-1}_{\text{substrate}}$, (b) [ii] time = 1 h and b [iii] temperature = 34.5°C .

The empirical models for predicting the degree of removal of arabinose side chains by recombinant AbfB and MeGlcA side chains by the purified AguA at any set of given hydrolysis conditions are presented based on coded values in Equations 6.3 and 6.4 respectively.

$$Z = 9.5 \cdot 10^3 + 4.0 \cdot 10^3 X_1 + 5.18 X_1^2 - 5.4 \cdot 10^2 X_2 - 4.1 \cdot 10^2 X_2^2 - 3.1 \cdot 10^3 X_3 + 2.1 \cdot 10 X_3^3 - 4.2 \cdot 10^3 X_1^2 X_2^2 + 1.5 \cdot 10 X_1 X_2^2 + 1.3 \cdot 10^3 X_1^2 X_2 - 5.5 \cdot 10^2 X_1 X_2 - 4.4 \cdot 10^2 X_1^2 X_3 + 37.84 \quad (6.3)$$

$$Z = 3.2 \cdot 10^2 + 20 X_1 - 60 X_1^2 - 22 X_2 - 95 X_2^2 + 72 X_3 - 40 X_3^2 - 45 X_1 X_2^2 - 6 X_1 X_2 + 8 X_1^2 X_3 + 30 X_1 X_3 - 53 X_1^2 X_2 + 6 X_2 X_3 \quad (6.4)$$

Where: Z = Predicted response (degree of side chain removal).

X₁, X₂, X₃, = Coded linear values for (1) hydrolysis time (h), (2) temperature (°C) and (3) enzyme xylan nkat substrate (g)⁻¹specific dosage or xylan and enzyme dosage.

X_{1,2,3}² = Coded quadratic values for (1) hydrolysis time (h), (2) temperature (°C) and (3) enzyme xylan nkat substrate (g)⁻¹specific dosage or xylan and enzyme dosage.

ε = Error

The significant main and interaction effects ($p < 0.05$) are presented in the Pareto charts for the recombinant AbfB removal of the arabinose side chains from the oat spelt xylan (Figure 6.3a (iv)) and the removal of MeGlcA side chains from the birch xylan by the purified AguA (Figure 6.3b (iv)). The significant linear effects for arabinose side chain removal by the recombinant AbfB in descending order, were hydrolysis time and recombinant AbfB xylan specific dosage (Fig. 6.3a (iv)). Whereas, the significant quadratic effects were temperature, hydrolysis time and recombinant AbfB xylan specific dosage (Fig. 6.3a (iv)). The significant interaction effects were mainly between the linear and quadratic effects of time and temperature, (Fig. 6.3a (iv)). On the other hand, the hydrolysis parameters with significant linear effects for the removal of MeGlcA side chains by the purified AguA the from birch xylan were in descending order, AguA xylan specific dosage, temperature and time whereas the significant quadratic effects included temperature and hydrolysis time (Fig. 6.3b (iv)).

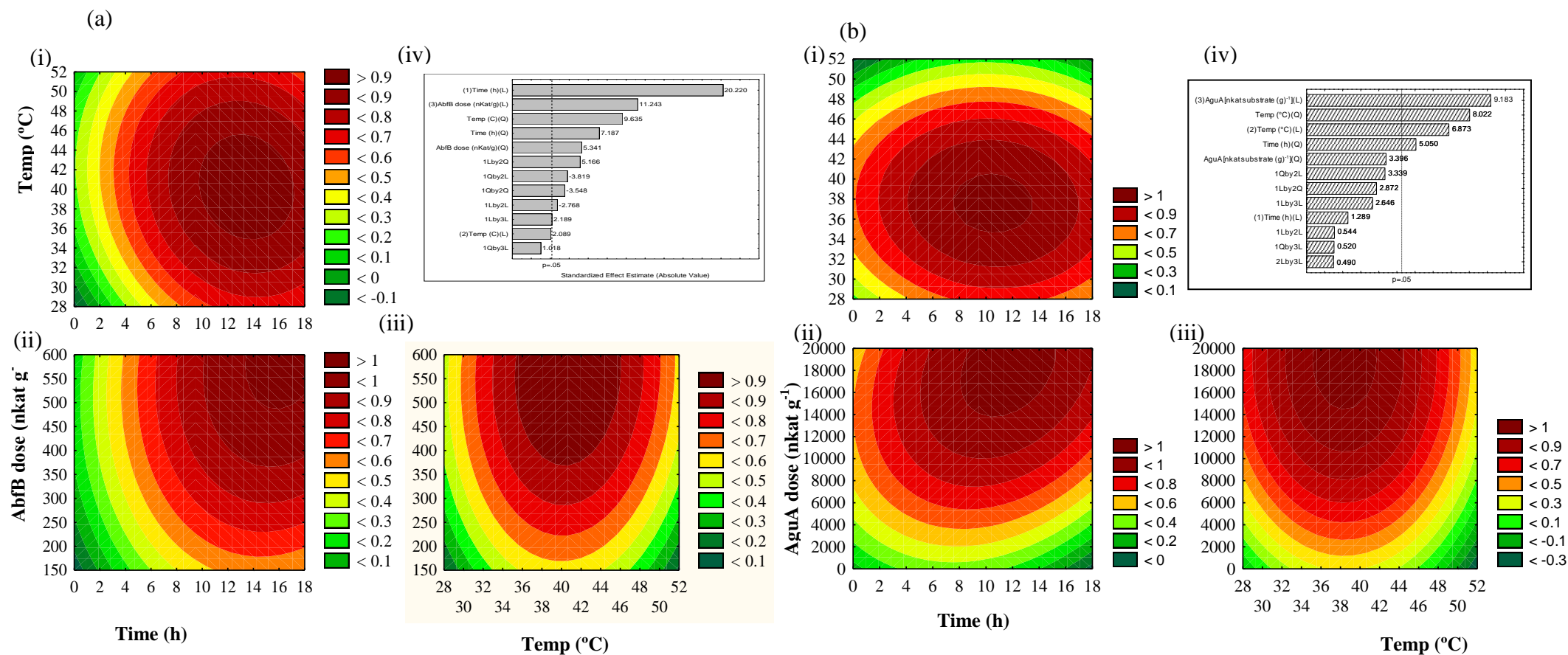


Figure 6.3: Desirability plot for determining optimal values of hydrolysis time, temperature, and enzyme dose for (a) arabinose and (b) MeGlcA side chains removal. Desirability plot (i) for temperature (Temp) versus time, (ii) enzyme dose (recombinant α -L-arabinofuranosidase (AbfB) / purified α -D- glucuronidase (AguA)) versus time, and (iii) enzyme dose (recombinant AbfB /purifiedAguA) versus temperature. Size and level of significance of the main treatment parameters and their interaction are shown in Pareto chart on (a) [iv] the removal of arabinose side chains from oat spelt xylan by recombinant AbfB and (b) [iv] removal of MeGlcA side chains from birch xylan by purified AguA. The L and Q in the Pareto charts denote linear and quadratic relationships. The $t_{(1, 14)}$ values are indicated at the end of each bar graph in the respective Pareto charts. The vertical dotted line in the Pareto chart is a measure of statistical significance at $p=0.05$.

Table 6.4: Regression coefficients for arabinose release as a function of time, temperature and enzyme xylan specific dose (coded values)

<i>Hydrolysis parameter</i>	Arabinose release = f (time, temperature, specific AbfB level) R^2 = 0.99 (R^2 adjusted =0.97)		Size of effect	Level of significance
	<i>Regression coefficient in polynomial equation</i>	<i>Regression coefficient value</i>	<i>t(2)</i>	<i>P value</i>
Mean/interaction	b_0	9476.69	29.01	<0.01
Main effects				
(1)Time (h)(L)	b_1	3989.39	19.94	<0.01
Enzyme dose (nkat/g substrate)(Q)	b_{33}	-3145.29	-5.34	<0.01
(3)Enzyme dose (nkat/g substrate)(L)	b_3	2071.60	7.32	<0.01
(2)Temp (°C)(L)	b_2	-540.89	-1.91	0.076
Temp (°C)(Q)	b_1	-405.20	-0.64	0.532
Time (h)(Q)	b_{11}	5.18	0.01	0.992
Interactions				
1Q by 2Q	b_{1122}	-4178.84	-3.55	<0.01
1L by 2Q	b_{122}	-1461.58	-5.17	<0.01
1Q by 2L	b_{112}	1323.20	3.82	<0.01
1L by 2L	b_{12}	-553.82	-2.77	<0.01
1L by 3L	b_{13}	437.94	2.19	<0.05
1Q by 3L	b_{113}	-352.67	-1.02	0.326
Error	E	37.84		

Note: 1 = time, 2 = temperature, 3 = enzyme xylan specific dosage, L = linear, Q = quadratic, R^2 = linear regression coefficient, $b_{i...ni}$ = model regression coefficient, letters and numbers in bold = significant.

Table 6.5: Regression coefficients for MeGlcA release as a function of time, temperature and enzyme xylan specific dose (coded variable)

MeGlcA release = f (time, temperature, specific AbfB level). $R^2 = 0.99$ (R^2 adjusted =0.94)				
			Size of effect	Level of significance
<i>Hydrolysis parameter</i>	<i>Regression coefficient in polynomial equation</i>	<i>Regression coefficient value</i>	<i>t(2)</i>	<i>P value</i>
Mean/Interaction.	b_0	317.43	24.28	0.002
Main effects				
(3)AguA [nkat substrate (g). l](L)	b_3	71.94	6.35	0.02
Temp (°C)(Q)	b_{22}	-94.53	-8.02	0.02
Time (h)(Q)	b_{11}	-59.51	-5.05	0.04
(2)Temp (°C)(L)	b_2	-22.36	-1.98	0.19
(1)Time (h)(L)	b_1	19.77	1.75	0.22
AguA [nkat substrate (g ⁻¹)(Q)	b_{33}	-40.02	-3.40	0.08
Interactions				
1L by 3L	b_{13}	29.96	2.65	0.12
1Q by 3L	b_{113}	8.33	0.52	0.65
1Q by 2L	b_{112}	-53.45	-3.34	0.08
1L by 2Q	b_{122}	-45.98	-2.87	0.10
1L by 2L	b_{12}	-6.16	-0.54	0.64
2L by 3L	b_{23}	5.55	0.49	0.67

Note: 1 =time loading, 2 = temperature, 3 = enzyme xylan specific dosage, L = linear, Q = quadratic, R^2 = linear regression coefficient, $b_1 \dots b_n$ = model regression coefficient, letters and numbers in bold = significant.

6.2.3 Effect of xylan concentration and enzyme dosage on removal of arabinose side chain from polymeric xylans.

The effects of varying xylan concentration and enzyme dosage as individual parameters were presented by a response surface plot (Fig. 6.4a). A maximum of $\approx 4\%$ arabinose (substrate basis or 40.% of the available arabinose) was achieved at xylan concentrations varied between 3000 and 6000 $\mu\text{g mL}^{-1}$ with AbfB volumetric activities between 17.5 and 37.5 nkat mL^{-1} (Fig. 4a). The desirability contour plot located the optimal xylan concentration to be 5600.00 $\mu\text{g mL}^{-1}$ and volumetric activity of the AbfB of 27.2 nkat mL^{-1} . At the such conditions, the recombinant AbfB arabinose removal was estimated to a maximum of 43% of the available arabinose (42700 $\mu\text{g g}^{-1}$ substrate) from oat spelt xylan was estimated (Fig. 6. 4b). The largest significant effect on the arabinose removal was from xylan concentration rather than the recombinant AbfB volumetric activity (Fig. 6.4c). The quadratic model for predicting the degree of the arabinose removal as a function of xylan concentration and enzyme dosage fitted the response surface plots with $R^2 = 0.89$ (adjusted $R^2 = 0.79$). The coefficients for the fitted response surface model (Equation 6.5) are presented in Table 6.

$$Z = 3.99 + 0.9X_1 - 0.84X_1^2 - 0.31X_2^2 + 0.22X_1X_2 \quad (6.5)$$

Where: Z = Predicted response (degree of arabinose side chain removal),
 X_1, X_2 , = Coded linear values for (1) xylan concentration ($\mu\text{g mL}^{-1}$) and
 (2) Enzyme dosage (nkat mL^{-1})

Table 6.6: Regression coefficients for arabinose release as a function of the xylan concentration and enzyme activity (coded variables).

Arabinose release = f (AbfB activity, Xylan concentration) ; $R^2 = 0.89$ (R^2 adjusted =0.80)				
<i>Hydrolysis parameter</i>	<i>Regression coefficient in polynomial equation</i>	<i>Regression value</i>	<i>coefficient</i>	<i>Level of significance P</i>
			<i>Size of effect $t(2)$</i>	<i>value</i>
Mean/interaction	b_0	3.99	11.62	<0.01
Main effects				
(1)Xylan conc. $\mu\text{g mL}^{-1}$ (L)	b_1	0.90	5.23	<0.01
(1)Xylan conc. $\mu\text{g mL}^{-1}$ (Q)	b_{33}	-0.835	-3.67	<0.01
(2)AbfB dose activity (nkat mL^{-1} substrate)(Q)	b_3	-0.314	-1.38	0.226
Interactions				
1L by 2L	b_{12}	-553.82	-0.91	0.41

Note: 1 = xylan concentration, 2 = enzyme activity, L = linear, Q = quadratic, R^2 = linear regression coefficient, $b_1 \dots b_n$ = model regression coefficient, letters and numbers in bold = significant at $P < 0.05$.

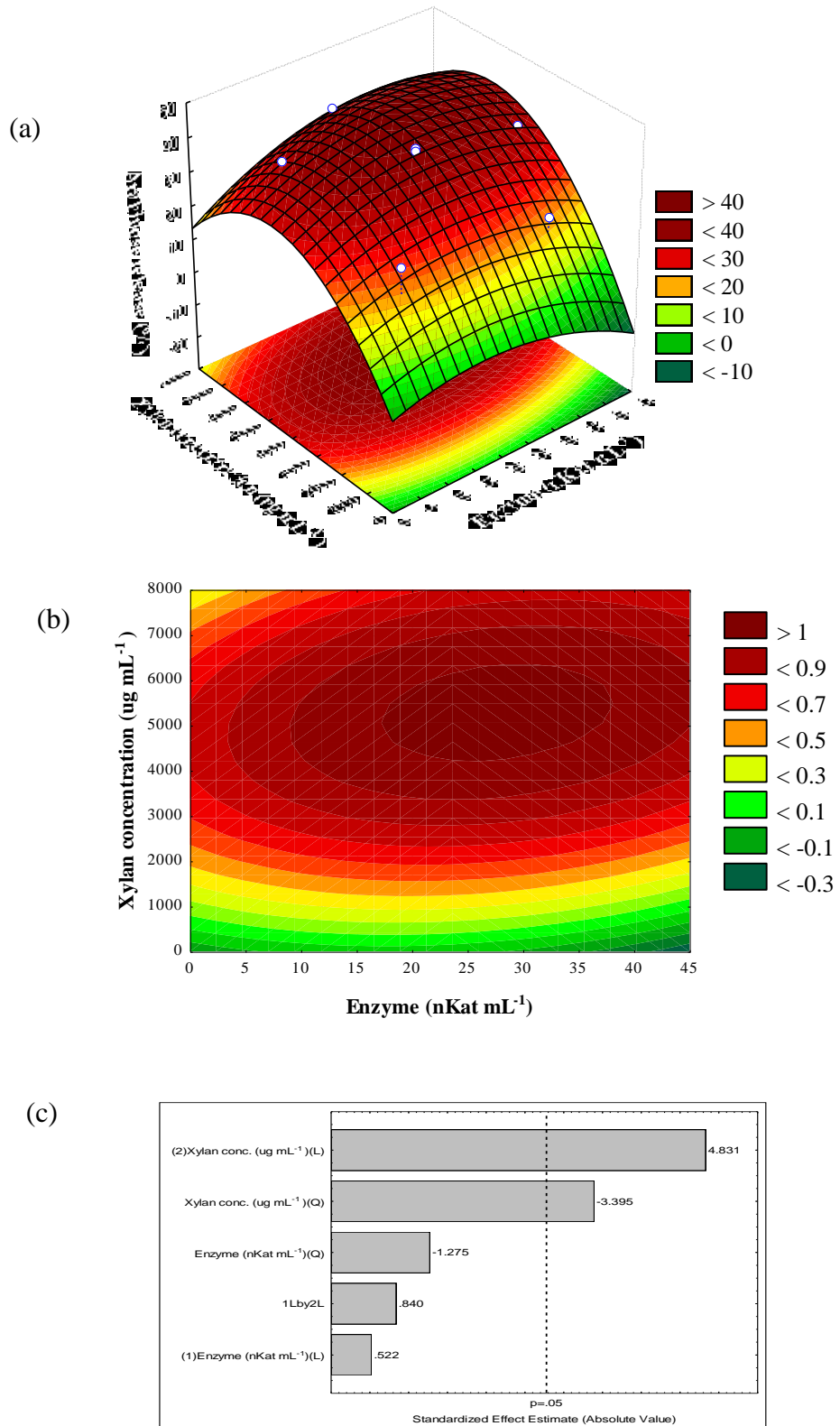


Figure 6. 4: Effect of arabinose removal by recombinant AbfB as a function of xylan concentration ($\mu\text{g mL}^{-1}$) and enzyme (AbfB) dose (nkat mL^{-1}) as reflected by (a) surface plot (b) The optimal levels are reflected in the desirability plot and (c) The size and the significance of the treatment and interaction effect the Pareto chart is measured by the bar graph. The $t_{(1,9)}$ values are indicated at the end of each bar graph. The vertical dotted line in the Pareto chart is a measure of statistical significance at $p = 0.05$. Hydrolysis was performed at 40°C for 16 h at pH 5.0.

6.3 DISCUSSION

6.3.1 Optimal conditions for enzymatic removal of arabinose and MeGlcA side chains from polymeric xylans

The optimal values for hydrolysis time, recombinant AbfB xylan specific dosage and temperature for maximum removal of arabinose side chain from oat spelt xylan were found to range from 10-16 h, temperature ranging from 35-45°C (Fig. 6.3a (i-iii) and enzyme xylan specific dosage of $607.0 \text{ nkatg}^{-1}_{\text{substrate}}$ (Fig. 6.4). At these conditions, an estimated maximum of 43% of the available arabinose side chains could be released from selective hydrolysis oat spelt xylan by the recombinant AbfB (Fig.6.4). These results are difficult to compare with results from other studies because optimisation of the enzymatic removal of arabinose side chains from polymeric xylans is rarely reported. In addition, enzymatic xylan hydrolysis parameters for removal of side chains such as hydrolysis time, temperature and enzyme dosage are often not optimized together. Nonetheless, the estimated maximum removal of the arabinose side chains by the recombinant AbfB was higher than that of α -L-arabinofuranosidase from *Rhizomucor pusillus* HHT-1, which removed 24% of the available arabinose but after 48 h of hydrolysis (Rahman et al., 2001) but was lower than that of α -L-arabinofuranosidases from *Thermobacillus xylanilyticus*, which removed 64% of the available arabinose after 50 h of hydrolysis (Debeche et al., 2000). The differences between the degrees of arabinose removal of this study and that from literature could, in part, be attributed to the differences in the hydrolysis conditions, in particular the enzyme to xylan dosage. However enzymatic hydrolysis that reaches maximum removal of the side chains for periods of up to 2 days (Rhaman et al., 2001; Debeche, et al., 2000) may not be practical for industrial applications.

Strategies for improving selective removal of arabinose side chains at optimal hydrolysis time, temperature and enzyme xylan specific dosage should bring about high efficiency in the enzymatic modification of xylans. The hydrolysis time, recombinant AbfB xylan specific dosage, temperature and the interactions of the hydrolysis time with temperature were significant ($p < 0.05$) and for the selective removal of the arabinose side chain from oat spelt xylan by the recombinant AbfB (Fig. 6.3a iv). Notable, the maximum degree of removal of arabinose side chain from the oat spelt xylan by the recombinant AbfB was achievable at a wider range of

hydrolysis time and AbfB xylan specific dosage compared to the temperature ranges (Fig. 6.3a (i-iii)). Therefore, increased degree of removal of arabinose side chain from the oat spelt xylan by the recombinant AbfB can efficiently be obtained at minimum optimal temperature by manipulating the hydrolysis time and the AbfB xylan specific dosage. The results in Fig. 6.1a clearly showed that the selective hydrolysis of oat spelt xylan by the recombinant AbfB at xylan specific dosage of $350.0 \text{ nkatg}^{-1}_{\text{substrate}}$ performed at 40°C increased the removal of arabinose side chain more than 3 times with increased hydrolysis time from 4 to 8 h. Similarly, increasing the AbfB xylan specific dosage from 350.0 to $700.0 \text{ nkatg}^{-1}_{\text{substrate}}$ for hydrolysis time of 4 h at 40°C doubled the release of the arabinose side chains from the oat spelt xylan by the recombinant AbfB (Fig. 6.1a). Furthermore, the results in Figure 6.2a (i-iii) showed that maximum liberation of the arabinose from the oat spelt xylan was achievable by the recombinant AbfB with various combinations of levels of hydrolysis time in the range between 10.8 and $>18\text{h}$ and AbfB xylan specific dosage between 427.0 and $>540.0 \text{ nkatg}^{-1}_{\text{substrate}}$ but the temperature range was restricted to between 35 and $\leq 45^{\circ}\text{C}$. The removal of arabinose side chains from the oat spelt xylan at such low temperatures would be energy efficient compared to physical and chemical processes that often are performed at high temperatures greater than 100°C (Linder et al., 2003).

The narrow temperature range for maximum removal of arabinose side chain from oat spelt xylan by the recombinant AbfB could possibly be a reflection of the thermo stability of both the AbfB and the xylan. The maximum optimal operating temperature for the recombinant AbfB was identified as 50°C (Chapter 4). Therefore, the selective hydrolysis of xylan performed at temperatures beyond 50°C would have direct consequences on the functioning of the recombinant AbfB. Most AbfBs showed rapid inactivation of AbfB of up to 95% beyond 60°C because such temperature is beyond the physiological threshold of the recombinant AbfB (Chapter 4). In this study, 93% reduction in selective removal of the arabinose occurred by performing hydrolysis of oat spelt xylan at enzyme xylan specific dosage of approximately $720.0 \text{ nkatg}^{-1}_{\text{substrate}}$ at 60°C compared to the hydrolysis performed at 40°C (Fig. 6.1b). Thermo instability of the recombinant AbfB at temperatures beyond 50°C is a typical property of fungal AbfBs belonging to family glycoside hydrolase (GH) 54 (Koseki et al., 2003; De Ioannes et al., 2000; De Wet et al., 2008). Therefore, effective removal of arabinose

side chain by recombinant AbfB should be performed where the AbfBs are thermostable.

The effect of changing hydrolysis time and enzyme xylan specific dosage on the AbfB removal from the oat spelt xylan would be associated mainly with the rate at which the AbfB accessed the side chains thereby reaching saturation state (Segel, 1976). Therefore, the Michaelis–Menten enzymatic kinetics, which the recombinant AbfB displayed (Chapter 4), suggest that its action would be affected by the oat spelt xylan concentration. This is because at saturation point, enzymes that follow the Michaelis–Menten enzymatic kinetics are known to stagnate despite prolonging the reaction or increasing the substrate dosage (Segel, 1976). The results showed that for the same degree of arabinose removal of 27% from oat spelt xylan the hydrolysis time was shortened from 16 h to 8 h by increasing the recombinant AbfB xylan specific dosages from 700.0 nkat $\text{g}^{-1}_{\text{substrate}}$ to 1 400.0 nkat $\text{g}^{-1}_{\text{substrate}}$ (Fig. 6.1a). Similarly, at lower levels (12-15%) of arabinose removal were observed at a lower enzyme specific dosage of 550 nkat $\text{g}^{-1}_{\text{substrate}}$ at 40.5°C and hydrolysis time of 16 h (Fig. 6.3a (i-iii)) compared to enzyme specific dosage of 607 nkat $\text{g}^{-1}_{\text{substrate}}$ that was identified as the optimal (Figure 6.4). For this reason, the ratio between the enzyme activity and the xylan concentration (enzyme specific dosage) is considered a limiting factor for obtaining higher degree of arabinose removal from the selective hydrolysis of the oat spelt xylan by the recombinant AbfB.

The optimal values of AguA xylan specific dosage, hydrolysis time and temperature of for maximum removal of MeGlcA side chain from birch xylan by the purified AbfB were 18 000 nkat $\text{g}^{-1}_{\text{substrate}}$, 38°C and 11 h respectively (Fig. 6.3b (i-iii)). At these conditions, an estimated maximum of MeGlcA side chains of 508.0 ug MeGlcA per unit substrate (g) (equivalent to 0.5% of available MeGlcA in the birch xylan) would be released. Tenkanen and Siika-aho (2000) reported that the AguA removed 50% of the MeGlcA side chains, utmost, by the purified AguA from deacetylated birch xylan (Roth) despite increasing hydrolysis time or enzyme xylan specific dosage. The enzymatic hydrolyses that involve large quantities of the recombinant AbfB without increasing the removal of the arabinose side chains would be costly for large scale xylan processing due to the limited availability of the enzyme (Chapter 4).

During the selective removal of MeGlcA side chains from birch xylan by the purified AguA, the largest significant effect ($p < 0.05$) was from the AguA xylan specific dosage compared to temperature and hydrolysis time (Fig. 6.3b (iv)). Therefore, the removal of the MeGlcA chains could be enhanced by increasing the enzyme dosage rather than temperature and time. The surface plots showed that maximum degree of MeGlcA side chains by the purified AguA could be achieved in 1 h (Fig. 6.3b (i-iii)). Unlike the removal of the arabinose side chain by recombinant AbfB from oat spelt xylan, the ratio between enzyme activity and xylan concentration was not optimized for maximum removal of MeGlcA from birch xylan due to logistical problems in obtaining the purified AguA. Furthermore, the interaction effects between hydrolysis time, temperature and AguA xylan specific dosage were not significant on the removal of MeGlcA from birch xylan by the purified AguA (Fig. 6.3b (iv)). A similar observation was evident from the results of Tenkanen and Siika-aho (2000) who showed that increasing both hydrolysis time (from 10 h to 24 h) and enzyme specific dosage of AguA (from 100 to 500 nkatg⁻¹_{substrate}) resulted in only a smaller difference in the degree of the removal of MeGlcA from birch xylan compared to separate increase in either hydrolysis time or AguA xylan specific dosage.

The optimal hydrolysis conditions identified for maximum removal of arabinose side chain and MeGlcA removal did not correspond to the conditions for obtaining maximum degree of precipitation of the xylans. The optimal conditions for release of arabinose from the oat spelt xylan were higher than the conditions at which maximum precipitation of the oat spelt xylan hydrogels and birch xylan occurred (Chapter 5). For instance, the results in Figure 6.4b showed that an estimated degree of removal of arabinose side chain by recombinant AbfB of up to 43% could be achieved under optimal conditions. Yet, the work presented in Chapter 5 suggested that increasing arabinose side chain removal beyond 10% of the available arabinose did not further increase the precipitation of the oat spelt xylan. Therefore, the degree of removal of both arabinose side chain by recombinant AbfB and MeGlcA by the purified AguA should be customized for the desired end use of the modified xylans.

The optimal range for values of hydrolysis time and temperature for AguA removal of MeGlcA from birch xylan (Fig. 6.3b (i-iii)) were within the same optimal range of the AbfB removal of arabinose side chain from oat spelt xylan (Fig. 6.3a (i-iii)), which

are hydrolysis time and temperature ranges of 8-16 h and 36-44°C respectively. These results indicate that the enzymological properties of the AbfB and AguA are from a thermostability point of view compatible for their simultaneous application in the selective hydrolysis of arabinoglucuronoxylans. The similar optimal values of hydrolysis time and temperature optimal values for AbfB (Fig. 6.3a (i-iii)) and AguA (Fig. 6.3b (i-iii)), suggest that synergetic removal of arabinose side chain by the recombinant AbfB and of MeGlcA side chains by the purified AguA from arabinoglucuronoxylan can be performed under optimal conditions for either of the enzymes. Ultimately, there are prospects for application of the two enzymes together on diversified range of lignocellulosic including the removal of arabinose side chain from arabinoglucuronoxylans extracted from the South African feedstocks (Chapter5).

6.3.2 Predicting degree of enzymatic removal of side chain from xylans

The experimental data for both removal of arabinose side chain by recombinant AbfB and the removal of MeGlcA by the purified AguA fitted the polynomial model with regression coefficient of > 0.90 . The regression analysis showed that hydrolysis time, temperature, AbfB xylan specific dosage and the interaction between hydrolysis time and temperature and between time and AbfB xylan specific dosage had significant effects on the removal of side chain from xylans and therefore, are important parameters for predicting degree of AbfB arabinose removal. For instance, the regression coefficients in the arabinose release prediction model showed that coefficients for linear effect of hydrolysis time, both linear and quadratic effect of enzyme xylan specific dosage were significant at $p < 0.01$ (Table 6.4). Furthermore, the coefficients for the interaction effects between hydrolysis time and enzyme specific xylan dosage were significant at $p < 0.01$ whereas the linear interaction between time and temperature were significant at $p < 0.05$. The regression analysis on the data from removal of arabinose side chain by recombinant AbfB from oat spelt xylan showed that degree of AbfB arabinose release from oat spelt xylan could be predicted by the quadratic model Equation 6.3 at any given combination of levels of the temperature, enzyme xylan specific dosage and time with a regression coefficients of $R^2 = 0.99$ (adjusted $R^2 = 0.97$). At optimal temperature and hydrolysis time, the model fitting the response surface plot showed that degree of removal of arabinose side chain by recombinant AbfB can be predicted from various combinations of xylan

concentrations and enzyme concentrations using Equation 6.5. The model fitted the response surface plot with regression coefficients of $R^2 = 0.89$ (adjusted $R^2 = 0.80$).

In AguA liberation of MeGlcA from birch xylan, the most important hydrolysis parameters for predicting degree of AguA liberation of MeGlcA were AguA xylan specific dosage and temperature. The regression analysis showed that the regression coefficients for linear effect of AguA xylan specific dosage, the linear and quadratic effects of temperature and the interaction of the linear effect of time and quadratic effect of temperature were significant at $p < 0.01$, but the regression coefficient of the linear effect of temperature was significant at $p < 0.05$. The model fitting the response surface for AguA removal of MeGlcA as a function of time, temperature and AguA xylan specific (Equation 6.4) yielded a linear regression coefficient ($R^2 = 0.99$ (R^2 adjusted = 0.94)).

6.4 CONCLUSION

The removal of the arabinose side chain from oat spelt xylan by the recombinant AbfB and the removal of MeGlcA side chains from birch xylan by the purified AguA were affected by variations in hydrolysis time, enzyme xylan specific dosage and temperature at different magnitudes. The optimal values of the hydrolysis parameters for removal of arabinose side chains from oat spelt xylan by the recombinant AbfB were 14-16 h at 38-45°C with recombinant AbfB xylan specific dosage of 607.0 nkat g⁻¹_{substrate}. Whereas, the optimal levels for removal of MeGlcA side chain from birch xylan were 11 h at 38°C with AguA xylan specific dosage of 18 000 nkat g⁻¹_{substrate}. Optimal conditions for the efficient and customized removal of the arabinose side chains from oat spelt xylan and of MeGlcA side chains from birch xylan by the recombinant AbfB and purified AguA respectively could be predicted by the quadratic models that fitted the response surface plot with regression coefficients > 0.9. The observed overlapping thermostability properties of the recombinant AbfB and purified AguA render the two enzymes capable of being used for synergetic modification of solubility properties of arabinoglucuronoxylans. The optimal values for maximum removal of the side chains from the xylan are different from the conditions for achieving maximum degree of precipitation of the xylan and formation of hydrogels, which indicates the need for customized removal of the side chains for specific end use of the modified xylan.

Chapter 7: *In situ* enzyme aided modification and adsorption of water soluble xylans onto cellulosic material

ABSTRACT

The effects on precipitation and adsorption of xylans onto the cotton lint of *in situ* selective removal of arabinose and MeGlcA side chains by recombinant α -L-arabinofuranosidase (AbfB) and purified α -D-glucuronidase (AguA) respectively were investigated. The cotton lint was treated with xylans extracted from bagasse, bamboo, *Pinus patula* and *Eucalyptus grandis* in the presence of either the recombinant AbfB or purified AguA or a cocktail of the two enzymes. Reference xylans included birch, oat spelt, H₂O₂ bleached bagasse and *E. grandis* xylan gel. The presence of the purified AguA increased the adsorption of xylans extracted from *E. grandis*, *P. patula* and bagasse by 334, 112 and 29% respectively, but decreased the adsorption of *E. grandis* xylan gel and H₂O₂ bleached bagasse xylan by 31 and 6% respectively. The recombinant AbfB increased the adsorption of xylans extracted from the bamboo, *P. Patula* and oat spelt by 31, 44 and 900% respectively, but decreased that of the bagasse and H₂O₂ bleached bagasse xylans by 13 and 30% respectively. The adsorption of *E. grandis*, *P. patula* and bamboo xylans increased the most in the presence of the AguA and AbfB. Furthermore, the adsorption of the xylans in the presence of the AbfB, AguA and a cocktail of the two enzymes, improved water holding characteristics and xylan–cellulose interactions onto the cotton lint suggesting the possibility of producing speciality cellulosic products with new and improved physical and functional properties in an integrated process with xylan extraction and pulping. However, the AguA was the most effective enzyme in increasing adsorption of the water soluble arabinoglucuronoxylans onto the cotton lint.

7.0 INTRODUCTION

Xylan is one of the major hemicelluloses present in cell walls of higher plants. The xylan exists in a matrix in close association with cellulose encrusted by lignin (Fengel and Wegener, 1989). The association of the xylan with the cellulose and lignin occurs both within and outside the cell wall, which presents a variety of opportunities to use the xylan to improve and introduce new functional properties in lignin and cellulosic materials. It is observed during the late stages of kraft pulping of wood that less substituted xylan fractions of higher molecular mass have a tendency to precipitate, aggregate and irreversibly adsorb onto the pulp fibres (Walker, 1965; Sjöström, 1993; Schönberg et al, 2001; Dahlman et al, 2003). The adsorption of xylans onto the pulp fibre surface was evident mainly when the alkalinity charge of the kraft cook liquor decreased, most probably due to the loss of side chains under the kraft cook conditions (Sjöström, 1993). The precipitation, aggregation and adsorption of the xylans onto cellulosic materials were influenced by the type, degree of substitution and substitution pattern of the side chain on the main xylan backbone (Linder et al., 2003^{ab}; Kabel et al., 2007). Other factors affecting the precipitation and aggregation of the xylans have been identified as xylan concentration (Chapter 6) and surface charge (Ebringerová and Heinze, 2000). For instance, rye arabinoxylan exhibited lower solubility resulting into aggregation behaviour when the arabinose to xylose ratio was less than 0.30 (Höije et al., 2008). Furthermore, Kabel et al. (2007) indicated that precipitation and adsorption of the xylans onto the cellulosic material occurred with presence of at least 15 consecutive unsubstituted xylose residues in the xylan backbone chain. Linder et al (2003^{ab}) indicated that adsorption of xylans onto cellulosic materials occurred with pre-aggregated xylans.

The adsorbed xylans provided the pulp fibres with improved drainage, which caused the fibres to swell leading to improved fibre to fibre bonding (Walker, 1965; Meller, 1965; Dahlman, et al., 2003; Håkansson et al., 2005) In addition, the adsorbed xylans introduced surface charges and form nano-sized hydrogel aggregates on the fibre surface (Henriksson and Gatenholm, 2001; Henriksson and Gatenholm, 2002^{ab}; Linder et al., 2003^a; Cha and Chinnan, 2004; Laganón et al., 2005; Kabel et al., 2007) . Such nanohydrogels can be used as implantation matrices onto cellulosic materials. Additionally, xylans are associated with medicinal properties that were identified to

be important for chiral separation, preparation of cholesterol depressants, tablet disintegrants, HIV inhibitors (e.g. inhibitor of growth of sarcoma-180 and other tumors) and anti-ulcer activity (Glasser, et al., 2000; Ebringerová, et al., 2002; Ciprian et al., 2008).

The natural ability of xylans to adsorb onto the cellulosic surface and improve the physical and functional properties of the cellulosic material make xylans better additives in lignocellulose processing materials compared to the commonly used additives, which include starch and clay. The additives of starch and clay origin suffer retention, dusting and cause weakening of cellulose fibre to fibre bonding (Maurer and Kearney 1998; Yan et al., 2005; Fras-Zemljič et al., 2006; Yoon, 2007; Nelson, 2007; Shen et al., 2009). Therefore, for the past decade, xylan-cellulose interactions have become the basis for novel research in the production of functional biomaterials in the food packaging, pharmaceutical and biomedical industries. For instance, xylan coating led to production of novel food packaging material with restricted gas permeability known as xylophane (Gröndahl et al. 2004; Gatenholm, et al., 2008) and production of xylan–cellulosic composites with reduced specific water transmission of up 362% (Saxena and Ragauskas, 2009).

Adsorption of the xylans onto the cellulosic material could be improved by processes that enhance formation and precipitation of insoluble hydrogels from the water soluble xylans without causing damage to the cellulosic material and the xylan main chain structure. Other workers have used various physical and chemical methods that induce precipitation and adsorption of the xylans onto the cellulosic fibres. In most cases, the conditions for precipitation and adsorption of the xylans onto cellulosic materials have imitated those of kraft cooking conditions (Temperature of $> 170^{\circ}\text{C}$ and alkalinity charge of $> 16\%$) (Gabrielii, et al., 2000; Jain, et al., 2001; Lindblad et al., 2001, Henriksson and Gatenholm, 2002^{ab}; Linder et al 2003^{ab}; Lindblad and Albertsson, 2005; Hettrich et al., 2006; Danielsson, 2007; Könhke et al., 2008). However, the reduction of solubility of xylans for adsorption onto cellulosic materials under such kraft cook like conditions could be damaging to both the xylans and cellulosic fibres. For instance, in kraft pulping of wood, xylan and cellulosic fibre losses of up to 50% occur due to random cleavage of the side groups and peeling effect (Sjöström, 1993). A similar trend is observed in kraft pulping of bamboo

whereby the pulp yield accounted to Kappa number 20 (Salmela et al., 2008). Consequently, the pulp yields and pulp fibre strength are reduced, which results in inefficient xylan adsorption process. Alternative to kraft cook conditions, other workers have used chemicals such as oxalic acid, acetic acid and sulphur dioxide (Walker, 1965, Westbye et al., 2006, Sternemalm et al., 2008) to reduce the solubility of xylan and form xylan hydrogel precipitates. Furthermore, physical methods including subjecting the water soluble xylans to supercritical temperature and pressure conditions have also been used as means of precipitating xylan (Haimer et al., 2008). However, similar to the kraft pulping conditions, the chemical and physical methods may lead to inefficient adsorption process and not provide the opportunity for customised precipitation and adsorption of the xylans onto the cellulosic material. Therefore, biological methods, in particular the use of side chain removing enzymes that selectively removed the side chains from polymeric xylans extracted from a wide variety of feedstock found in South Africa (Chapters 4-6), appear to be attractive alternative tools for customised precipitation and adsorption of xylans onto cellulosic materials.

The purpose of the study was to assess the effectiveness of using the recombinant α -L- arabinofuranosidase (AbfB) (EC 3.2.1.55) (Chapter 4) and purified α -D-glucuronidase (AguA) (EC 3.2.1.139/131) described by Tenkanen and Siika-aho (2000) for simultaneous *in situ* precipitation and adsorption of xylans isolated from *Eucalyptus grandis*, *Pinus patula*, *Bambusa balcooa* and bagasse (Chapter 3) onto model cellulosic material (cotton lint). In the study, the precipitation and adsorption of reference xylans including oat spelt, birch, H₂O₂ bleached bagasse and *E. grandis* xylan gel were also assessed. A process flow diagram illustrating integration of the simultaneous enzymatic aided *in situ* xylan precipitation and adsorption onto cellulosic materials during pulping of lignocellulosic materials was proposed.

7.1 MATERIALS AND METHODS

7.1.1 Materials

The study used arabinoglucuronoxylans extracted from *Saccharum officinarum* L (sugarcane) bagasse, *Bambusidae balcooa* (bamboo) and *Pinus patula* and glucuronoxylan derived from *Eucalyptus grandis*, using a mild alkali method (Chapter 3). Reference xylans included: commercial oat spelt xylan (Sigma, # 0627), birch xylan (Roth, #7500), mild alkali H₂O₂ bleached xylan from bagasse (generously donated by Prof. A.M.F. Milagres, School of Engineering, University of São Paulo, Brazil) and a *E. grandis* xylan gel (kindly supplied by Dr A. Bayley of SAPPI, Pretoria, South Africa). The cellulosic fibre material was non-absorbent cotton lint (Grade 1, Cotton King). The arabinose side chain removing enzyme was recombinant α -L-arabinofuranosidase (AbfB) with volumetric activity of 18.0 nkat mL⁻¹ on *p*-nitrophenyl-arabinofuranoside (*p*NPA) produced in-house using recombinant *Aspergillus niger* D15 (Chapter 4). The MeGlcA side chain removing enzyme was purified α -D-glucuronidase (AguA) with specific activity of 300 nkat mg⁻¹ from wild type *Schizophyllum commune* (VTT-D-88362-ATCC 38548), generously donated by Prof. Matti Siika-aho of VTT Biotechnology Institute in Finland.

7.1.2 Xylan adsorption onto cellulosic material

Xylan solutions (1% w/v) were prepared according to De Wet et al. (2008). The cotton lint samples (1 g) were autoclaved in 100 ml Schott bottles at 121°C for 15 min. After cooling to room temperature, the oat spelt xylan solutions (1% w/v) 5, 12.5, 15.0 and 25 mL and AbfB (18.0 nkat mL⁻¹) were added in a fixed xylan to enzyme ratio of 5:2. The negative control xylan adsorption mixtures contained no enzyme while the positive control contained the enzyme but no cotton lint. The xylan adsorptions onto the cotton lint were performed in 0.05 M citrate buffer pH 5.0 at 40°C for 24 h in a 40 mL reaction volume. All reactions were terminated by placing the bottles in ice.

In another experiment, cotton lint samples weighing 0.2 g were placed in 15 mL glass bottles in two sets of three and a control. The bottles were autoclaved at 121°C for 15 min. Upon cooling to room temperature, 750 μ L birch xylan (1% w/v) and 150 μ L α -D-glucuronidase (AguA) (900 nkat mL⁻¹) were added to one set. Subsequently, 0.05M

acetate buffer (pH 4.8) was added to make a total reaction mixture of 1650 μL . The negative and positive controls consisted of the birch xylan in the absence of AguA and in the absence of cotton lint respectively.

The effect of the cocktail of recombinant AbfB and purified AguA on precipitation and adsorption of the xylans onto the cotton lint was tested in another experiment. The cotton lint (0.2 g) was treated in the xylan solutions prepared from mild alkali pre-extracted arabinoglucuronoxylans from bagasse, bamboo and *P. patula* in the presence of a cocktail of recombinant α -L-arabinofuranosidase and α -D-glucuronidase in glass bottles.

The reaction mixture of 2 mL contained 0.2 g cotton lint, 1 mL xylan (1% w/v), 0.1 mL α -L-arabinofuranosidase (AbfB) ($18.0 \text{ nkat mL}^{-1}$) and 0.04 mL α -D-glucuronidase (AguA) (900 nkat mL^{-1}) and 0.86 mL 0.05M acetate buffer pH 4.8. The reaction was performed at 40°C in a water bath for 16 h (Table 7.1).

7.1.3 Analysis of physical properties and chemical composition of the cotton lint

Treated cotton lint samples were vacuum-filtered and subsequently washed by suspending it in 50-100 mL Milli-Q H_2O while agitating for 1 h to disengage xylan precipitates loosely absorbed to the cotton lint. The Milli-Q H_2O was changed three times during the washing process. The samples were after the final wash, vacuum filtered in pre-weighed conditioned filter papers and placed in pre-weighed foils for drying overnight at 30°C to a constant weight (the drying temperature was chosen to avoid any structural changes to the adsorbed xylan). The amount of xylan adsorbed onto the cotton lint was defined as the difference between the initial weight of the cotton lint and the weight after the xylan treatment presented as a percentage of the initial weight of the cotton lint. Xylan specific weight gain for the cotton lint was defined as the weight gained by the cotton lint as a percentage of the initial amount of xylan in the reaction mixture. The calculations were corrected for moisture content of the starting materials and cotton lint losses during post treatment. The individual enzyme effect on xylan adsorption onto cotton lint was defined as the difference in weight gain between cotton lint treated with unmodified xylan and cotton lint treated in presence of the xylan.

Table 7.1: Experimental set up for treatment of cotton lint with xylan in the presence of α -L-arabinofuranosidase (AbfB), α -D-glucuronidase (AguA) and their cocktail (AG).

Xylan type [*]	Control ^{**}	Agua (900 nkat mL ⁻¹) 40 μ L ^{***}	AbfB (18.0 nkat mL ⁻¹) 100 μ L ^{****}	AbfB and AguA 100 μ L + 40 μ L ^{*****}
Bagasse H1	SBH1	GBH1	ABH1	AGBH1
Bagasse H2	SBH2	GBH2	ABH2	AGBH2
BagasseBraz1	SBB1	GBB1	ABB1	AGBB1
BagasseBraz2	SBB2	GBB2	ABB2	AGBB2
Bamboo1	SBM1	GBM1	ABM1	AGBM1
Bamboo2	SBM2	GBM2	ABM2	AGBM2
Pine 1	SP1	GP1	AP1	AGP1
Pine 2	SP2	GP2	AP2	AGP2
Eucalyptus H1	SEH1	GEH1		
Eucalyptus H2	SEH2	GEH2		
Eucalyptus gel S1	SES1	GES1		
Eucalyptus gelS 2	SES2	GES2		

* Xylan type prepared in duplicates (1 and 2), The BH, BB, BM, P, EH and ES denote mild alkali extracted xylan from bagasse, H₂O₂ bleached bagasse, mild alkali extracted bamboo, *Pinus patula*, *Eucalyptus grandis* and *Eucalyptus grandis* gel respectively. **Cotton lint treated with unmodified xylan (S), *** Cotton lint treated with xylan in presence of purified α -glucuronidase (G), **** Cotton lint treated with xylan in presence of recombinant α -arabinofuranosidase (A), ***** Cotton lint treated with xylan in presence a cocktail of purified α -glucuronidase and recombinant α -arabinofuranosidase(AG).

Carbohydrate composition of the adsorption mixture and the cotton lint were determined from xylan adsorption reaction mixture filtrates, which were centrifuged at 10,000 rpm for 5 min at 4°C followed by filtration using 0.22 μ m pore size filters. The filtrates were analysed for L-arabinose and α -D MeGlcA release using HPAEC-PAD (Dionex) on CarbopacTM PA 10 column. The L-arabinose (Merck) and D-MeGlcA (Sigma) were used as reference sugars. Samples from xylan adsorption mixture filtrates were analysed following a phenol-sulphuric assay described by Dubois (1956). Analytical grade of xylose sugar (Merck) was used as a standard. Precipitation efficiency was defined as the amount of xylan removed from the adsorption reaction mixture as a percentage of the initial amount of xylan in the adsorption mixture (xylan in this case was measured in the form of xylose sugar).

Xylan adsorbed onto the cotton lint was analysed by a two- step sulphuric acid hydrolysis method. Samples of cotton lint treated with xylan, weighing 0.050 g, were hydrolysed in 0.5 mL of 72% H₂SO₄ in McCartney bottles followed by incubation in a water bath at 30°C for 1h. The reaction mixture was diluted to 4% by addition of 15 mL de-ionised water (dH₂O). The samples were autoclaved at 121°C for 1 h. The hydrolysates were vacuum filtered using glass microfiber filters followed by filtration using 0.22 µm pore size filter discs before sugar analysis. The xylose content in the cotton lint hydrolysate was determined using HPAEC-PAD (Dionex) on CarbopacTM PA 10 column. The efficiency of xylan adsorption onto the cotton lint due to enzymatic treatment of the xylan during the adsorption was defined as the difference between the amount of xylose in the hydrolysate of cotton lint treated in the presence and absence of the enzymes corrected for any xylose that pre-existed in the untreated cotton lint. Synergetic effect was defined as the difference between the amount of xylose released from acid hydrolysate of cotton lint treated in xylan adsorption mixtures in the presence of a cocktail of AbfB and AguA and the amount of xylose released the presence of AbfB or AguA, which was presented as a percentage.

7.1.4 The cotton lint solid state (CP/MAS) NMR analysis

Structural changes of the dried cotton lint samples as a result of xylan adsorption were analysed using solid state (CP/MAS) NMR on a Varian VNMRS 500 wide bore solid state NMR spectrometer with an operating frequency of 125 MHz for ¹³C using a 6mm T3 probe with a probe temperature of 25°C. Dry cotton lint samples were loaded to fill 6 mm zirconium oxide rotors. Spectra were recorded using cross-polarisation and magic angle spinning (CP/MAS). The speed of rotation was 5 kHz, the proton 90° pulse was 5 µs, the contact pulse was 1500 µs and the delay between repetitions of 5 s. Chemical shifts were determined relative to tetramethylsilane (TMS) by setting the downfield peak of an external adamantane reference to 38.3 ppm. Assignment of the spectra was according to Atalla and Isogai (2005).

7.1.5 Statistical analysis:

Unless stated otherwise, samples were conducted in triplicates. The experimental data were processed using analysis of variance (ANOVA) and regression analysis using Microsoft Excel and Statistica 2007 software programmes.

7.2 RESULTS

7.2.1 Sugar profile of cotton lint and adsorption mixture after xylan adsorption

The acid hydrolysates of the cotton lint post oat spelt xylan adsorption in the presence of recombinant AbfB at xylan dosage levels of 25 and 12.5 mL g⁻¹ contained 8 and 4% xylose respectively (Fig. 7.1a). Whereas, the corresponding acid hydrolysate of the cotton lint treated with oat spelt xylan in the absence of the recombinant AbfB at xylan dosage levels of 25 and 12.5 mL g⁻¹ contained 6 and 0.4% xylose respectively (Fig. 7.1a). The reduction of xylose content in adsorption mixtures was 64% (representing degree of xylan precipitation) after xylan adsorption in the presence of the recombinant AbfB at both 25 and 12.5 mL g⁻¹ xylan dosage levels (Fig. 7.1b). The xylose reduction in oat spelt xylan mixture hydrolysed by the AbfB in the absence of the cotton lint was 25 and 42% for the xylan dosage levels of 25 and 12 mL g⁻¹ respectively (Fig. 7.1b). About 30 and 50% of the available arabinose side chains were released during the *in situ* AbfB oat spelt xylan hydrolysis and adsorption onto the cotton lint at 25 and 12.5 mL g⁻¹ xylan dosage levels respectively (Fig. 7.1b). Whereas, 60% of the arabinose was released during the AbfB hydrolysis of the oat spelt xylan in the absence of the cotton lint at both xylan dosage levels of 25 and 12.5 mL g⁻¹ (Fig. 7.1b).

The sugar profile of the acid hydrolysate of the cotton lint treated with birch xylan in the presence of AguA showed 1.21% xylose content whereas the one treated in the absence of the AguA had 0.77% xylose content (Fig. 7.1c). The xylose content of the birch xylan adsorption mixture decreased by 32% after adsorption of birch xylan onto the cotton lint in the presence of AguA, whereas that of birch xylan adsorption mixture in the absence of the AguA decreased by 28% after adsorption onto the cotton lint (Fig. 7.1c). The AguA released 7.9% MeGlcA during the adsorption of the birch xylan onto the cotton lint (Fig. 7.1c).

The sugar profile of the cotton lint hydrolysates after adsorption of arabinoglucuronoxylans extracted from bagasse, bamboo and *P. patula* in the presence of the recombinant AbfB, purified AguA and a cocktail of the two enzymes is presented in Figure 7.1d. The xylose contents of the cotton lints treated with the

bagasse xylan in the presence of purified AguA, cocktail of the recombinant AbfB and purified AguA, recombinant AbfB and in the absence of the enzyme were 2.0, 1.6, 1.4 and 1.6% respectively (Fig. 7.1d). The xylose content of the hydrolysate of the cotton lint treated with the H₂O₂ bleached bagasse xylan in the absence of the enzymes was the highest (2.0%) (Fig. 7.1d). The lowest xylose content was found in the acid hydrolysate of the cotton lint treated in the presence of the recombinant AbfB (Fig. 7.1d). The xylose content of the acid hydrolysate of the cotton lint treated with bamboo xylan in the presence of the recombinant AbfB, purified AguA and a cocktail of the AbfB and AguA was 1.4, 1.8 and 1.5% respectively (Fig. 7.1d) whereas, the xylose content of the hydrolysate of the cotton lint treated with bamboo xylan in the absence of the enzymes was the lowest accounting for 1.0% (Fig. 7.1d). The xylose contents of the acid hydrolysate of cotton lint treated with *P. patula* xylan in the presence of the purified AguA, recombinant AbfB and AbfB and AguA cocktail and in absence of both enzymes were 1.3, 1.1, 1.01 and 0.61% respectively (Fig. 7.1d).

The sugar composition of the acid hydrolysate of cotton lint treated with glucuronoxylans extracted from *E. grandis* and *E. grandis* xylan gel in the presence and absence of purified AguA is shown in Figure 7.1d. The acid hydrolysate of the cotton lint treated with *E. grandis* in the presence of the purified AguA contained about 1.94% xylose whereas the acid hydrolysate that was treated with *E. grandis* xylan in the absence of the purified AguA enzyme contained 0.45% xylose (Fig. 7.1d). The acid hydrolysate of the cotton lint treated with *E. grandis* xylan gel showed a contrasting trend of the xylose content. The acid hydrolysate of the cotton lint that was treated with *E. grandis* xylan gel in the presence of the purified AguA had a lower xylose content (4.0%) compared to 5.7% that was present in the acid hydrolysate of cotton lint treated with the *E. grandis* xylan gel in the absence of the purified AguA (Fig. 7.1d).

7.2.2 Cotton lint xylan specific weight gain

The cotton lint showed weight increase after xylan adsorption. The cotton lints specific xylan weight gains as a result of individual and combined application of the AbfB and AguA during adsorption of arabinoglucuronoxylans

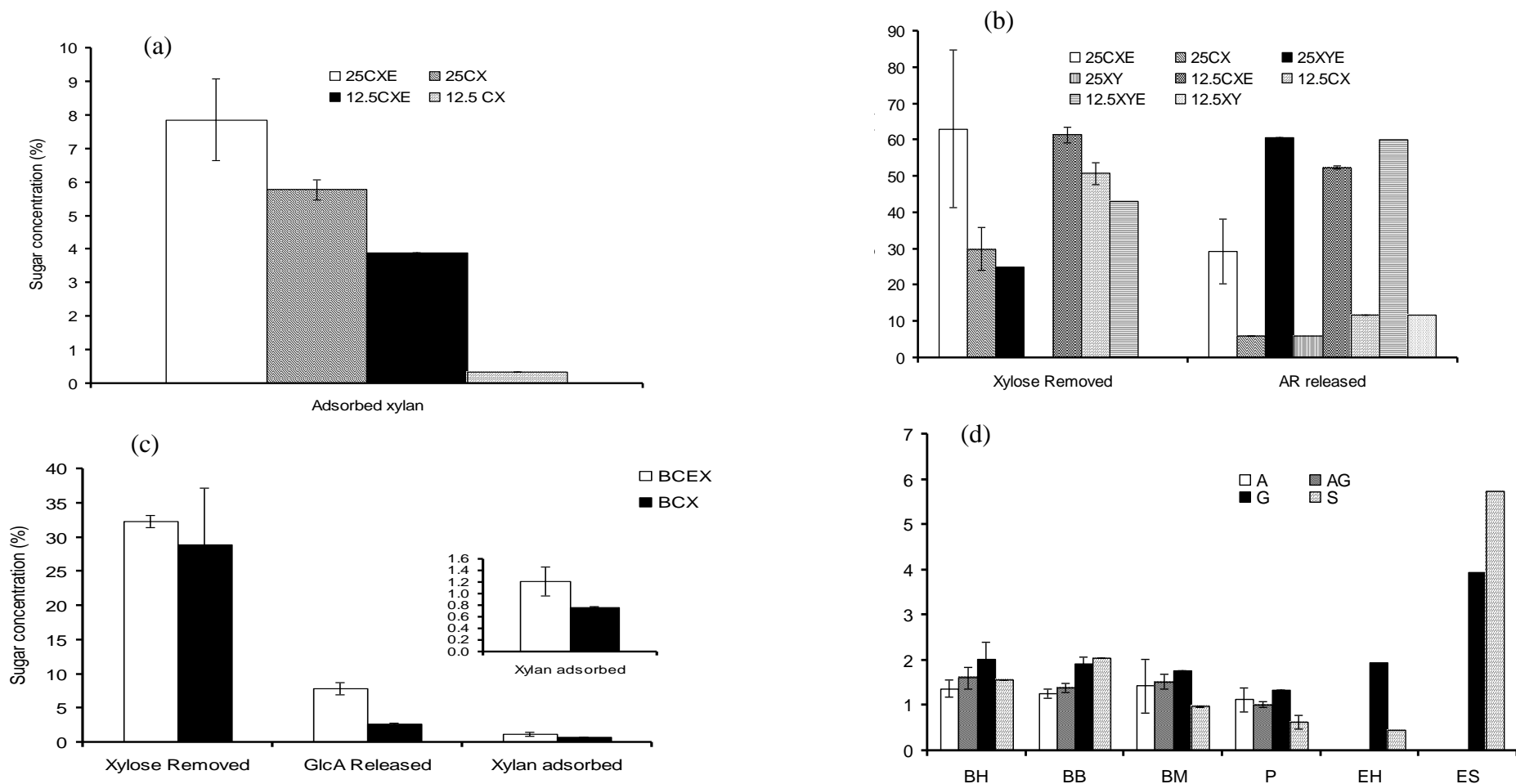


Figure 7.1: Sugar composition (a) of cotton lint after treatment with oat spelt xylan in the absence (CX) and in the presence of AbfB (CXE) at xylan dosage level of 12.5 and 25.0 mL g⁻¹, (b) sugar profile of the xylan adsorption mixture after cotton lint treatment in the absence (CX) and presence of AbfB (CXE), in absence of the cotton lint and AbfB (XY) and in the presence of AbfB without the cotton lint (XYE) at a dosage levels of 12.5 and 25 mL g⁻¹. (c): sugar profile of cotton lint and adsorption mixture after treatment with birch xylan in absence (BCX) and presence of AguA (BCXE) and (d) of cotton lint treated with bagasse (BH), H₂O₂ bleached bagasse (BB), bamboo (BM), *P. patula* (P), *E. grandis* (EH) and *E. grandis* gel (ES) in the presence of AbfB, AguA and AbfB/AguA denoted by prefices A, G and AG and in the absence of any enzyme denoted by prefix S.

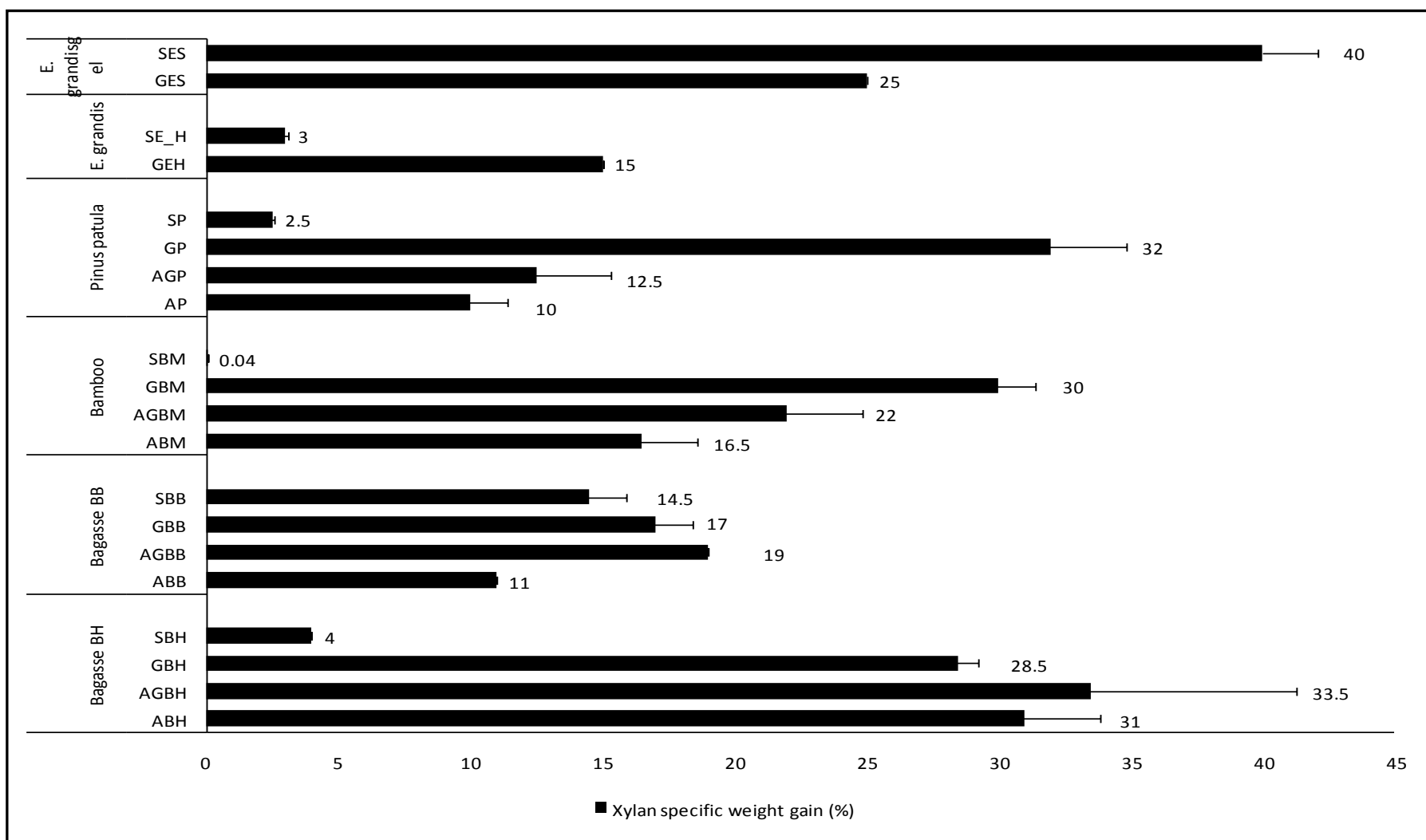


Figure 7.2: Cotton lint xylan specific weight gain after adsorption of pre-extracted xylans from bagasse (BH), H_2O_2 bleached bagasse (BB), bamboo (BM), *Pinus patula* (P), *Eucalyptus grandis* (EH) and *Eucalyptus grandis* gel (ES) in the presence of recombinant α -L-arabinofuranosidase (AbfB (A)), purified α -D-glucuronidase (AguA (G)) and a cocktail of recombinant AbfB and purified AguA. The prefix S denotes treatment with xylan in the absence of enzymes, BH and EH denotes xylans extracted by mild alkali using the Hoije method (Chapter 4). The bar graph represents the means and standard deviations of duplicate samples.

extracted from bagasse were in decreasing order of 34, 31, 29 and 4% for adsorption in the presence of AbfB and AguA cocktail, recombinant AbfB, purified AguA and in the absence of these two enzymes respectively (Fig. 7.2). The specific xylan weight gain of the cotton lint treated with H₂O₂ bleached bagasse xylan in the presence of recombinant AbfB, purified AguA, cocktail of the two enzymes and in the absence of both enzymes was less than 20% (Fig. 7. 2). However, the highest cotton lint xylan specific weight gain of 19% was observed with cotton lint that was treated with H₂O₂ bleached bagasse xylan in the presence of a cocktail of the AbfB and AguA and the lowest (14%) was for the cotton lint treated with H₂O₂ bleached bagasse xylan in the presence of recombinant AbfB (Fig. 7. 2). The xylan cotton lint specific weight gains after adsorption of bamboo xylan was the highest (30%) for the adsorption that was performed in the presence of both the recombinant AbfB and the purified AguA followed by the adsorption performed in the presence of the cocktail of the AbfB and AguA (Fig. 7. 2). Similar to cotton lint treated with bamboo xylan, the specific xylan cotton lint weight gains was the highest (32%) for the cotton lint that was treated with *P. patula* xylan in the presence of purified AguA (Fig. 7. 2). The specific xylan weight gain for the cotton lint treated with *E. grandis* xylan in the presence of purified AguA was 15%, whereas in its absence it was 3% (Fig. 7.2). In contrast, the xylan specific weight gain for cotton lint treated with *E. grandis* xylan gel in the presence and absence of the purified AguA was 25 and 40% respectively (Fig. 7.2).

The cotton lint xylan specific weight gain for cotton lint that was treated with oat spelt xylan at dosage levels of 5, 12.5, 15 and 25 mL g⁻¹ in the presence of recombinant AbfB was 42, 26 and 22% respectively (Fig. 7.3). In the absence of the recombinant AbfB, the xylan specific weight gain of the cotton lint treated with oat spelt xylan for dosage levels of 12.5 mL g⁻¹ and 25 mL g⁻¹ was approximately 27 and 18% respectively (Fig. 7.3). The cotton lint treated with birch xylan in the presence and absence of α -D-glucuronidase at birch xylan dosage level of 3.75 mL g⁻¹ had a specific xylan weight gain of 16 and 7% respectively (Fig. 7.3).

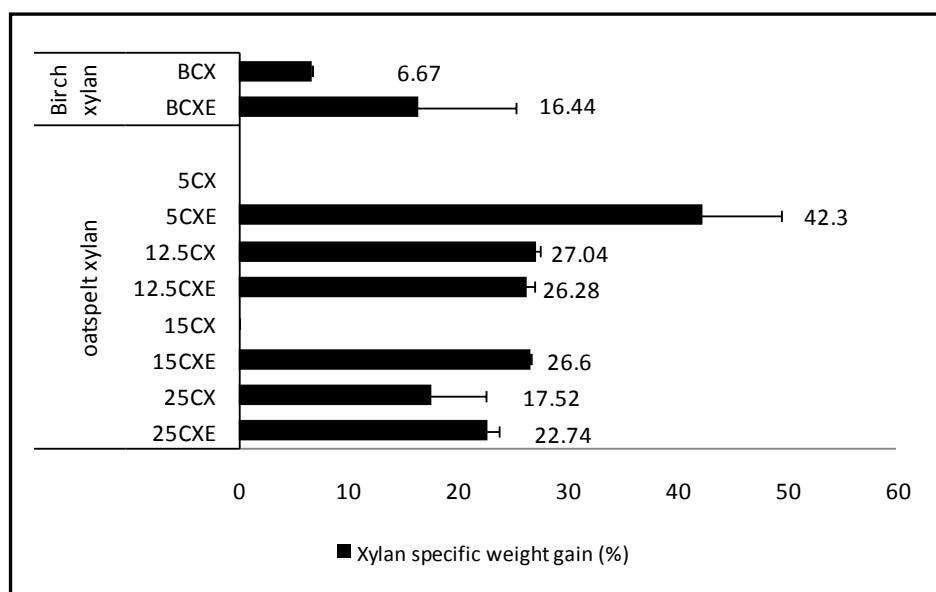


Figure 7.3: Cotton lint xylan specific weight gain after adsorption of oat spelt and birch xylan. Bar graphs indicate means and standard deviations of duplicate samples. The CXE denotes cotton lint treated with oat spelt xylan in presence of AbfB. BCXE is cotton lint treated with birch xylan in the presence of AguA. CX denotes cotton lint treated with oat spelt xylan in the absence of AbfB, whereas, BCX denotes cotton lint treated with birch xylan in the absence of AguA. The numbers 5, 12.5, 15 and 20 indicate oat spelt xylan dosages in mL g⁻¹ of the cotton lint. Data for 5CX and 15CX were not included due to loss of samples.

7.2.3 ¹³C-(CP/MAS) NMR characterisation of cotton lint structural changes after treatment with enzymatically modified xylans

The solid state ¹³C-(CP/MAS) NMR spectra of the cotton lints on which enzymatically modified xylans were adsorbed, displayed signals for carbon resonances for glucose units associated with C-6 of the primary alcohol group at chemical shifts (δ) between δ 60 and 70 ppm (Figs. 7.4a-h). The C-2, C-3 and C-5 ring carbons of the glucose units other than those anchoring glycosidic bonds, appeared between δ 70 and 81 ppm (Figs. 7.4a-h). In addition, the signals for carbon resonances associated with C-4 appeared in the region between δ 81-93 ppm, whereas those associated with anomeric carbon (C-1) were displayed between δ 102 and 108 ppm (Figs. 7.4a-h). Furthermore, the spectra displayed signals assigned to crystalline cellulose between δ 88-89 ppm and around 65 ppm (Figs. 7.4a-h). The signals assigned to amorphous region of cellulose were identified at between δ 83-84 ppm and 61-62 ppm (Fig. 7.4a-h).

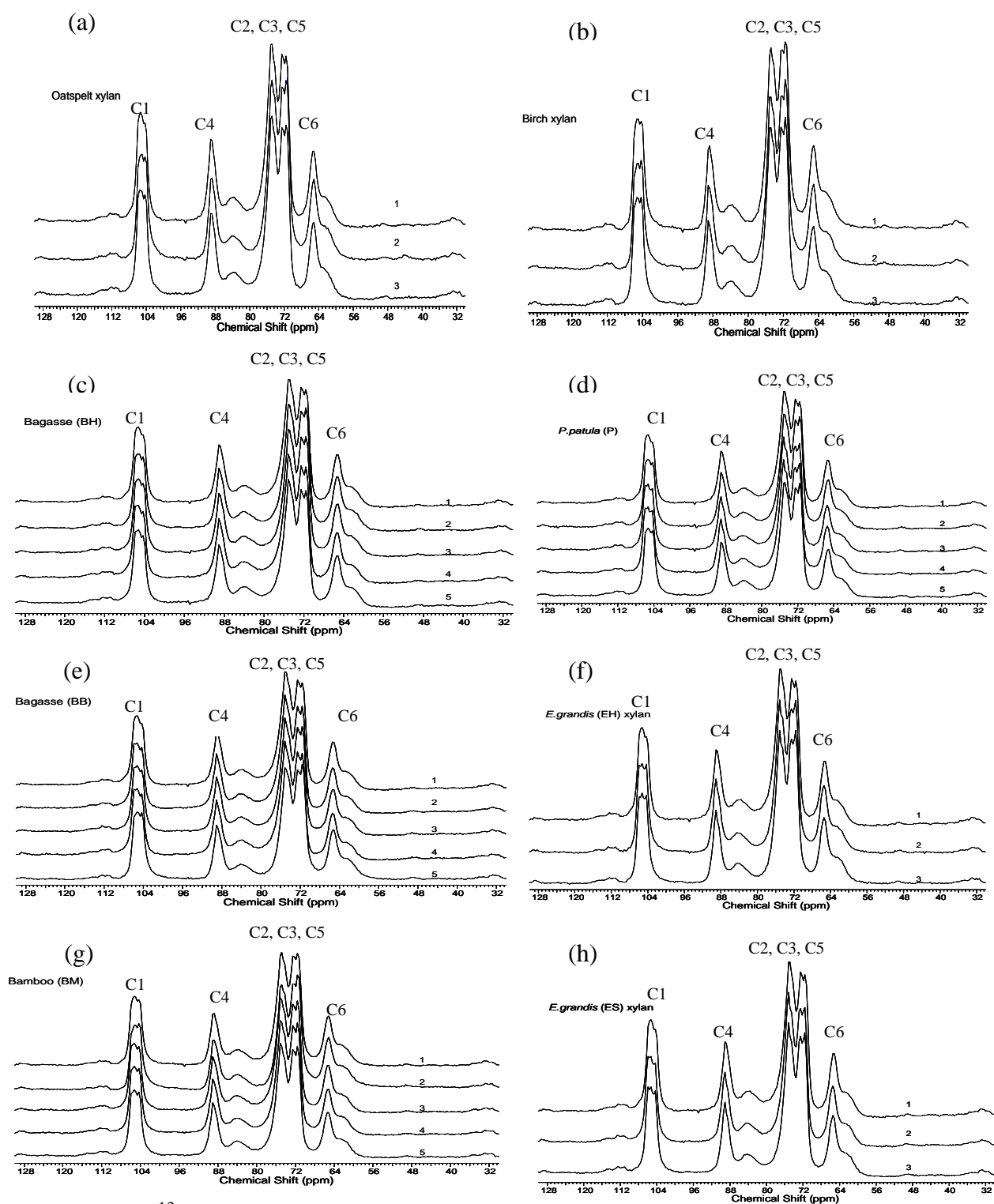


Figure 7.4: ^{13}C -CP/MAS-NMR spectra for cotton lint treated with (a) oat spelt xylan (b) birch xylan (c) bagasse (BH), (d) *P. Patula* (BB), (e) H_2O_2 bleached bagasse (f) *E. grandis* (EH) xylan, (g) bamboo (BM), and (h) *E. grandis* gel (ES). In the spectra (a) and (b) [1] denotes untreated cotton lint [2] cotton lint treated with xylans in the absence of enzymes [3] cotton lint treated with xylans in presence of either α -glucuronidase (AguA) or α -L-arabinofuranosidase (AbfB). In spectra (c)-(h), [1] denotes untreated cotton lint, [2] cotton lint treated with xylans in absence of enzymes [3] cotton lint treated with xylans in presence of AguA [4] treated with xylans in presence of AbfB /AguA cocktail and [5] treated with xylans in presence of AbfB.

There were changes in line sharpening and decrease of the intensity of C4 (δ 83-84 ppm) and C-6 (δ 61-62 ppm) upfield resonances in the solid state ^{13}C -(CP/MAS) NMR spectra of cotton lint treated with the xylans in presence of AbfB, AguA and a cocktail of the two enzymes (Figs. 7.4a-h). According to Atalla and Isogai (2005) the specified regions are associated with the amorphous cellulose present in the cotton lint (Figs. 7.4a-h, spectra 3-5). The change in splitting patterns of carbon resonances was observed in the anomeric carbon (C-1) resonances identified between δ 102 and 108 ppm in the solid state ^{13}C -(CP/MAS) NMR spectra for cotton lint treated with oat spelt xylan in the presence of the recombinant AbfB (Fig. 7.4a). Similar changes in the splitting patterns of the C-1 resonance were observed in the solid state ^{13}C -(CP/MAS) NMR spectra of the cotton lint treated with xylan from birch *E. grandis* and *E. grandis* xylan gel in the presence of purified AguA (Figs. 7.4b, f and h) and in the solid state ^{13}C -(CP/MAS) NMR spectra of the cotton lint treated with xylans from bagasse, *P. patula*, H_2O_2 bleached bagasse and bamboo both in the presence and absence of the AbfB and AguA (Figs. 7.4c, d, e and g).

7.2.4 Enzyme aided xylan adsorption in an industrial set up

(a) Integration of xylan extraction, precipitation and adsorption onto pulp fibres with pulping

A process flow diagram for application of the purified AguA and recombinant AbfB in precipitation and adsorption of xylans onto cellulosic surfaces is proposed in Figure 7.5. The xylan extraction is integrated with *in situ* enzymatic precipitation and adsorption of the xylan onto pulp fibres in a kraft pulp and paper making process. The diagram shows that xylans can be extracted from raw materials that are used for production of pulp prior to the pulping process. The extracted xylans are (*in situ*) within the processing line, enzymatically precipitated to enhance its adsorption onto the pulp fibres. The re-introduction of the xylans together with the side chain removing enzymes into the pulp and paper making processes can take place either before or after the bleaching stages.

(b) Mass balance of xylan in extraction, kraft pulping and adsorption processes

The xylan mass balance for an integrated process of xylan extraction from *E. grandis*, kraft pulping the residue and adsorption of the extracted xylan onto the cellulosic material is presented in Figure 7.6. The composition of *E. grandis* and the process conditions for the

integrated process are presented in Table 7.2. From a tonne of *E. grandis* a yield of water soluble xylan of about 70 kg is expected (Fig. 7.6) by using the Hoiye mild alkali extraction method (Chapter 3). The extracted xylan contains 31% acid insoluble lignin (Klason lignin) and 12.8% MeGlcA (Table 7.2). The composition of the cellulolignin obtained as a residue from the xylan extraction would contain 450 kg cellulose (Table 7.2). Pulping the cellulolignin residue under standard kraft pulping conditions would give 50% pulp yield (Ragauskas et al., 2006), which would contain 350 kg cellulose, 7.30 kg xylan and 7.30 kg lignin (Fig. 7.6). Subjecting the cellulosic material to xylan adsorption in the presence of AguA (1.9 kg) at 10 kg xylan per tonne of cellulosic material and adsorption efficiency of 2% (Fig. 7.1), the xylan coated cellulosic material of 364.66 kg is obtained (Fig. 7.6). The content of the cellulosic material is estimated to have 7.35 kg xylan. A balance of 68.9 kg of water soluble xylan and 0.03 kg MeGlcA are obtained (Fig. 7.6).

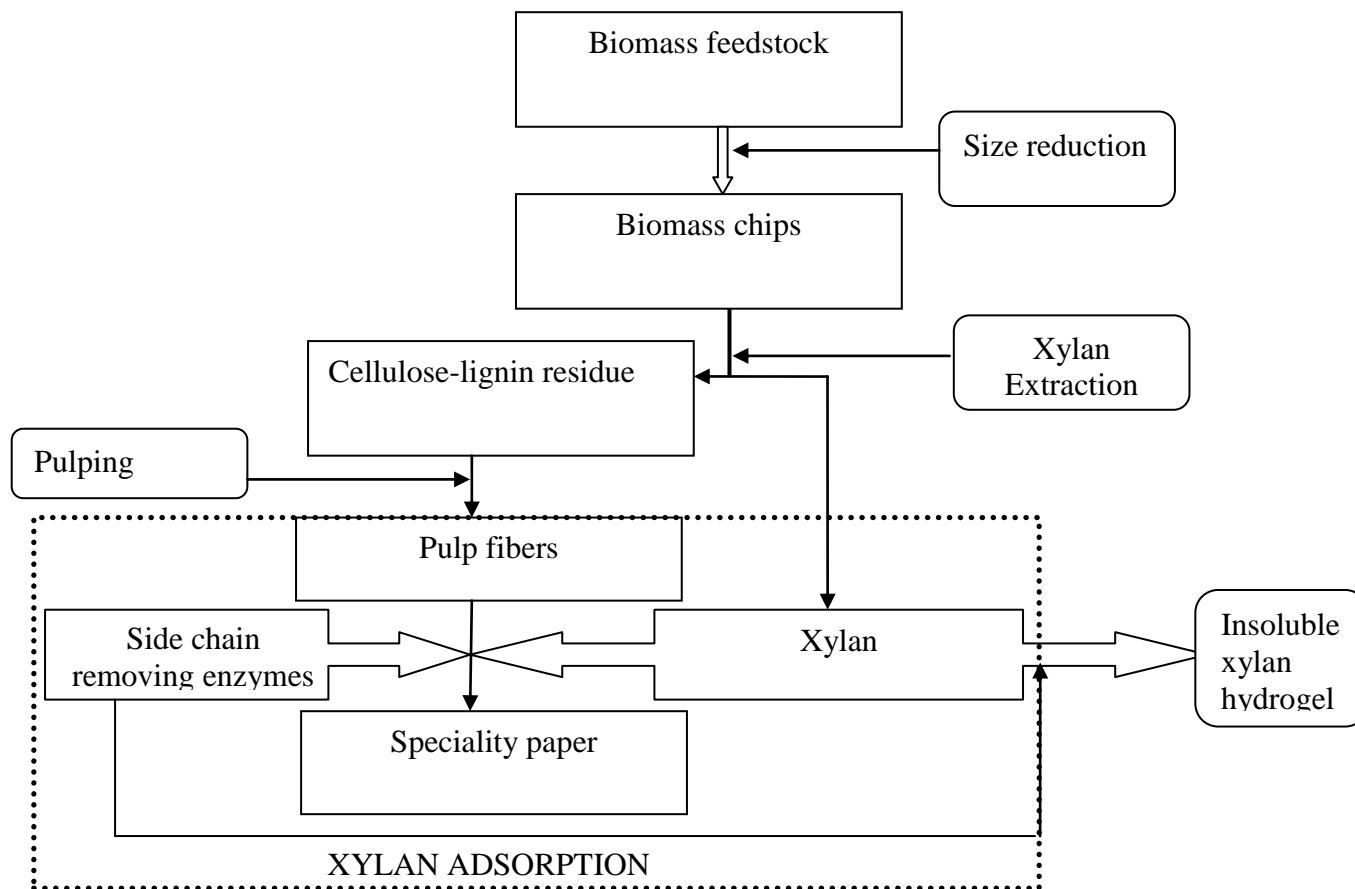


Figure 7.5: Illustration of integration of xylan extraction and re-introduction of the xylan in the presence of enzymes to allow adsorption onto pulp fibres during kraft pulp and paper making process.

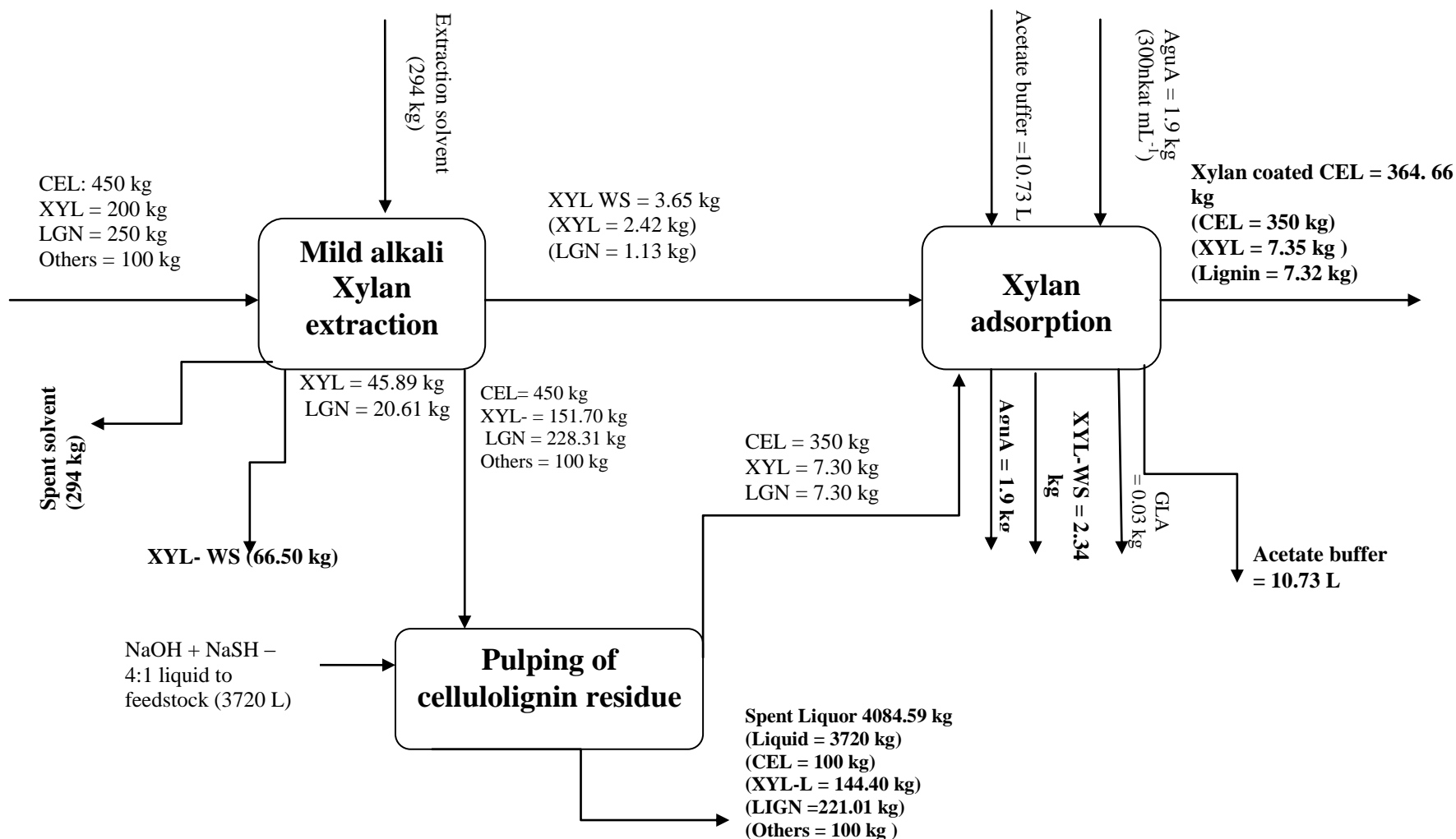


Figure 7.6: Xylan mass flow balance in an integrated process of xylan extraction, pulping, precipitation and xylan adsorption on cellulosic material per tonne of *Eucalyptus grandis*. Conditions for xylan extraction were described in Chapter 3, Kraft pulping conditions were described by Sjöström (1993) and conditions for xylan precipitation and adsorption onto cellulosic materials were 40°C and pH 4.8 as shown in Table 7.1. Abbreviations in the flow diagram are defined in Table 7.2.

Table 7.2 Xylan mass balance information for integrated enzyme aided precipitation and adsorption of xylan on cellulosic material in kraft pulping

Process	Composition per tonne of <i>E. grandis</i>
Composition of <i>E. grandis</i>	<ul style="list-style-type: none"> • 45% Cellulose (CEL) • 20% Xylan (XYL) • 25% Lignin (LGN) • 10% Others
Xylan extraction	<ul style="list-style-type: none"> • 35% Water soluble xylan (XYL-WS) yield • 69% xylan • 31% lignin
<u>Cellulolignin residue (Solid recovered)</u>	<ul style="list-style-type: none"> • Cellulose = 450 kg (assume 100% recovery from xylan extraction) • Xylan = 151.17kg • Lignin = 228.31 kg • Others = 100.00 kg
Pulping cellulolignin residue	<ul style="list-style-type: none"> • 50% pulp yield <ul style="list-style-type: none"> ○ Cellulose = 350.00 kg ○ Xylan = 7.30 kg (2% xylan of pulp yield) ○ Lignin = 7.30 kg (2% lignin of pulp yield)
Pulping residues	<ul style="list-style-type: none"> • Cellulose = 100 .00kg • Xylan = 144.40 kg • Lignin = 221.01 kg • Others = 100.00 kg
Xylan adsorption (input)	<ul style="list-style-type: none"> • Water soluble xylan 3.5 kg (10 kg/ tonne cellulose) <ul style="list-style-type: none"> ○ Xylan = 2.42 kg (69% XYL- WS, 12.8% MeGlcA (GLA) ○ Lignin = 1.08 kg (31% XYL-WS) • Cellulosic material = 364.60 kg <ul style="list-style-type: none"> ○ Cellulose = 350 kg ○ Xylan = 7.30 kg (present in fibre) ○ Lignin = 7.30 kg (present in fibre) • α-glucuronidase = 1.9 kg (removes 9% of GLA)
Xylan adsorption (output)	<ul style="list-style-type: none"> • Coated cellulosic material = 364.67 kg (2% xylan adsorbed) <ul style="list-style-type: none"> ○ Cellulose = 350 kg ○ Xylan = 7.35 kg ○ Lignin = 7.32 kg • Recovered from adsorption <ul style="list-style-type: none"> ○ Xylan- WS = 2.34 kg, GLA = 0.03 kg ○ Lignin = 1.06 kg ○ α-glucuronidase = 1.9 kg (300 nkat mL⁻¹)
Xylan extracted balance	<ul style="list-style-type: none"> • Xylan-WS = 66.35 kg <ul style="list-style-type: none"> ○ Xylan = 45.89 kg ○ Lignin = 20.61 kg

7.3 DISCUSSION

7.3.1 Effect of *in situ* selective enzymatic side chain removal on xylans adsorption

The AbfB and AguA have provided a simplified and high efficient process for enhancing adsorption of xylans onto cellulosic materials. For example, the adsorption onto cotton lint of arabinoglucuronoxylan extracted from *P. patula* of South African origin (Chapter 3) was increased by up to 80, 112 and 64% respectively, with *in situ* selective hydrolysis of the xylans by the recombinant AbfB, purified AguA and the mixture of these two enzymes (Fig. 7.1d). Although Linder et al. (2003^{ab}) reported successful controlled adsorption of birch xylan onto cellulosic material under kraft pulping like conditions, the removal of the side chains under kraft conditions occurs at random and is often accompanied by depolymerisation of the xylan main chain (Sjöström, 1993). Hence, the *in situ* simultaneous enzymatic precipitation and adsorption of the xylans would likely make more precipitated xylans available that can readily precipitate onto the cellulosic materials.

The mass balance for integrated xylan extraction, precipitation and adsorption onto the cellulosic material (Fig. 7.6) shows that from a tonne of *E. grandis* feedstocks, 70 kg of xylan could be extracted (at 35% extraction efficiency) but only 3.65 kg of the xylan is required for AguA enzymatic aided adsorption onto cellulosic materials at a xylan dosage level of 10 kg xylan per tonne of cellulosic material in which 2% of the xylan is adsorbed onto the cotton lint in the presence of 1.9 kg AguA (300 nkat mg⁻¹). In which case, up to 95% of the xylan extracted from the feedstocks could be spared for other uses. In consideration of the specific amounts of xylan required for adsorption, xylan dosage level for efficient adsorption of oat spelt in the presence of the recombinant AbfB should be below 25.5 mL g⁻¹. Therefore, an efficient *in situ* enzyme aided simultaneous precipitation and adsorption of the xylan onto cellulosic material should be designed based on efficient use of the xylans, the enzymes and energy.

7.3.2 Effect of xylan dosage on enzyme aided xylan adsorption onto the cotton lint

The xylan dosage level was one of the critical factors for efficient enzyme aided adsorption of oat spelt xylan onto the cotton lint. The adsorption of xylan at different dosage levels, assessed only with selective removal of arabinose from oat spelt xylan by the recombinant AbfB, was almost 30 times more efficient at 12.5 mL g⁻¹ xylan dosage level relative to 25 mL g⁻¹ xylan dosage level at the same given enzyme to xylan ratio of 5:2 (Fig. 7.1a). The results showed that the application of the recombinant AbfB increased the adsorption of oat spelt xylan onto cotton lint by up to 900% with oat spelt xylan dosage level of 12.5 mL g⁻¹ compared to 33% increase in adsorption achieved with 25 mL g⁻¹ xylan dosage level (Fig. 7.1a). Hence, the results of this study suggest that there should be an optimal xylan dosage for efficient xylan adsorption onto the cotton lint. Although Köhnke and Gatenholm (2007) attributed the increased adsorption of xylan onto pulp fibres to high xylan concentration, Kabel et al. (2007) indicated that the adsorption of xylan onto cellulosic materials is more efficient at lower xylan concentrations. In this study, the adsorption of the xylan at the lower xylan dosage was linked to the higher probability of the xylan hydrogel precipitates getting in contact with the cellulosic surface than at higher xylan dosage (Kabel et al., 2007).

The process of adsorbing xylans onto the cellulosic material could as well be related to the basic Langmuir theory that describes monolayer adsorption from a kinetic rather than thermodynamic point of view (Duong, 1998). The Langmuir theory suggests that there is a continuous bombardment of molecules on to the surface that correspond to loss of molecules from the surface to maintain a zero net accumulation. The fundamental principles of irreversible adsorption of polymers described in detail by Méndez-Alcaraz et al. (1998) indicate that the adsorption of on surfaces creates a thermally mobile layer giving rise to interactions that can be either attractive or repulsive. Linder et al. (2003^a) hypothesized that the xylans do not build up on the cellulosic surface with time by multilayer adsorption but rather by attachment of preformed aggregates of xylans, increases both in amount and size. Therefore, it might not be necessary to generate large amounts of xylan precipitates in excess of

what would cover the intended cellulosic surface area. For this reason, identifying the minimum required xylan dosage for adsorption onto the cellulosic material would improve the efficiency of the enzyme aided adsorption process.

7.3.3 Comparative effect of side chain removing enzymes on adsorption of arabinoglucuronoxylans onto cotton lint

The individual application of the purified AguA increased the adsorption onto the cotton lint of arabinoglucuronoxylans extracted from bagasse, bamboo and *P. patula* more than the application of the recombinant AbfB as well as the cocktail of the recombinant AbfB and the purified AguA. The xylose content of the acid hydrolysate of the cotton lint treated with arabinoglucuronoxylan extracted from bagasse, bamboo and *P. patula* and selectively hydrolysed *in situ* by the purified AguA, was 1.2-1.5 times that of the cotton lint that was treated *in situ* by the recombinant AbfB as well as the cocktail of the AbfB and AguA (Fig. 7.1d). The higher xylose content of the acid hydrolysate of the cotton lint was a reflection of the higher degree of adsorption of the arabinoglucuronoxylans onto the cotton lint in the presence of the purified AguA.

The higher performance of the purified AguA over the recombinant AbfB in aiding arabinoglucuronoxylans adsorption, presents the AguA as the enzyme of choice for enhancing the adsorption of the arabinoglucuronoxylans onto cellulosic materials. It appears that the selective removal of the MeGlcA side chain from the arabinoglucuronoxylans by the purified AguA provided the arabinoglucuronoxylans with more binding power to adsorb onto the cellulosic materials compared to the removal of the arabinose side chains by the recombinant AbfB. The conditions that favour xylan precipitation and adsorption of xylans onto cellulosic materials have been described by Kabel et al. (2007) to include existence of a region of at least 15 unsubstituted xylose units within the xylan main chains. Such unsubstituted regions in the xylan main chain are responsible for the electrostatic attraction forces within and between the xylan (Ebringerová and Heinze, 2000) that lead to increased adsorption on cellulosic materials. It implies that the selective removal of the MeGlcA by the purified AguA probably created large enough regions of unsubstituted xylose units in arabinoglucuronoxylans that enhanced the adsorption of the xylans onto cellulosic material than the removal of arabinose by the recombinant AbfB.

On the other hand, the limited adsorption of extracted from bagasse, bamboo and *P. patula* onto the cotton lint with *in situ* selective hydrolysis in the presence of the cocktail of the recombinant AbfB and the purified AguA is perhaps a reflection of the removal of the of the arabinose side chains from the xylan main chain may have restricted the selective removal of the MeGlcA side chains by the purified AguA from the arabinoglucuronoxylans , which reduced the affinity of the xylans to adsorb onto the cotton lint. Such phenomenon was explained in Chapter 5 and 6 in the context of synergetic effects of combined application of the two enzymes.

The limited individual action of the recombinant AbfB in enhancing adsorption of the arabinoglucuronoxylans onto the cotton lint compared to the purified AguA could probably be linked to substrate specificity limitations. Although the AbfBs are generally known to have broader xylan substrate specificities than the AguAs, some of the AbfBs are only capable of liberating the arabinose side groups attached to terminal non- reducing xylose units but fail to liberate the arabinose residues linked to *O*-2 of internal or non-reducing terminal xylose and arabinose adjacent to MeGlcA groups (Numan and Bhosle, 2006; Luonteri et al., 1998). The internal *O*-2 arabinose substitution patterns are a common feature in heteroxylans such as those derived from bagasse and bamboo (Timell, 1967; Wilkie, 1979; Ebringerová and Heinze, 2000). In this study, the internal *O*-2 arabinose substitution patterns were evident in the NMR spectra of the arabinoglucuronoxylans extracted from bamboo and bagasse (Chapter 3). Therefore, the results may imply that the recombinant AbfB may not have been able to detach the internally substituted arabinose side chains on the main xylan chain, which could have created the needed unsubstituted xylose regions for adsorption. Such an effect renders the recombinant AbfB to be a less effective enzyme for enhancing adsorption of the arabinoglucuronoxylans onto cellulosic materials compared to the purified AguA.

7.3.4 Effect of xylan source on enzyme aided xylan adsorption onto the cotton lint

The *E. grandis* and *P. patula* feedstocks present better sources of xylan for use in *in situ* enzyme aided adsorption of the xylans onto cellulosic materials than bagasse and bamboo. The results have shown that the increase in adsorption of xylans derived from wood sources including *E. grandis* and *P. patula*, adsorbed onto the cotton lint

in the presence of the recombinant AbfB, purified AguA and the cocktail of the recombinant AbfB and AguA was more than the increase in adsorption of xylans derived from grasses *vis* bagasse and bamboo. The increases in adsorption onto the cotton lint of xylans extracted from *E. grandis* (334%) and *P. patula* (112%) (Fig. 7.1d) were over 10 times the increase in adsorption of bagasse and bamboo xylans that was achieved in the presence of the AguA (Fig. 7.1d). Both the *E. grandis* and *P. patula* xylan fractions contained higher levels of MeGlcA sugar of approximately 13 and 12% (dry weight) respectively, compared to 9 and 11% (dry weight) of the MeGlcA content of bagasse and bamboo arabinoglucuronoxylan fractions (Chapter 3). Relative to the xylose content, the MeGlcA degree of substitution of *E. grandis* and *P. patula* xylan fractions was estimated to be 16 and 19% respectively and of bagasse and bamboo arabinoglucuronoxylan fractions to be 12 and 14% respectively. Such differences in the degree of MeGlcA side chain substitution on the main xylan chain between the wood and grass derived xylans could be attributed to the higher adsorption onto the cotton lint of the arabinoglucuronoxylans fractions from *P. patula* in the presence of the side chain removing enzymes compared to the arabinoglucuronoxylan fractions from bagasse and bamboo. This is because the xylan extracted from *P. patula* had similar degree of arabinose side chain substitution (25%) as the bagasse xylan fractions but differed in MeGlcA side chain substitution (Chapter 3). The difference in lignin content of the xylan fractions could have played a role in the degree of adsorption of xylans. Xylan fractions of high lignin content are known to adsorb more than their counterparts (Linder et al. 2003^a). The results showed that the *P. patula* xylan fractions had the highest acid insoluble residues compared to the bagasse xylan fractions, a property that was confirmed with the solid state NMR spectra (Chapter 3). However the effect of lignin content of the xylan fractions per se was not investigated in this study.

The glucuronoxylans extracted from *E. grandis* were better substrates for AguA aided xylan adsorption onto the cotton lint than the *P. patula* arabinoglucuronoxylans. Yet, the *P. patula* arabinoglucuronoxylans had a higher degree of MeGlcA substitution compared to *E. grandis* xylan (Chapter 3). The effect of substitution pattern on xylan adsorption could probably explain the observed effect. Kabel et al. (2007) reported that adsorption of xylans onto cellulosic materials could depend on the substitution pattern rather than degree of substitution alone. Indeed the results in Figure 7.1d

suggest that removal of MeGlcA from *E. grandis* glucuronoxylans resulted in higher xylan binding power towards the cotton lint than the removal of MeGlcA from the *P. patula* arabinoglucuronoxylans. Furthermore, Kabel et al. (2007) reported higher adsorption of glucuronoxylans, in particular, deacetylated xylans from Eucalyptus than arabinoglucuronoxylans from wheat to be linked to the differences the block wise substitution arrangement of the MeGlcA side chains on the main xylan chain of the glucuronoxylans such as those from *E. grandis* compared to the randomized substitution that prevails in most arabinoglucuronoxylans (Wilkie, 1979; Ebringerová and Heinze, 2000; Jacobs et al., 2001; Kabel et al., 2007). The increases in the *in situ* adsorption of the arabinoglucuronoxylans extracted from *P. patula* onto the cotton lint in the presence of the AbfB and AguA enzymes were higher than the increases in adsorption of up to 32% reported for corn cob arabinoglucuronoxylans onto softwood pulp (Ramírez et al., 2008). In addition, the increase in adsorption of 112% of the arabinoglucuronoxylan extracted from *P. patula* onto cotton lint in the presence of purified AguA exceeded the increase in adsorption of up to 80% achieved with chemically cationised xylans from barley husks onto bleached softwood pulp (Könhke et al., 2009). Therefore, the increased adsorption of arabinoglucuronoxylans onto cellulosic materials could also be linked to either the structure of the xylan fraction or the substitution pattern of the side chains after enzymatic hydrolysis.

The phenomenon of substitution pattern influencing xylan adsorption onto the cotton lint could also explain the limited adsorption behaviour displayed by the bagasse xylan fractions onto the cotton lint in the presence of the purified AguA when more MeGlcA side chains were removed from them than from the xylan extracted from bamboo (Chapter 5) (Fig. 7.1d). Therefore, although the bamboo arabinoglucuronoxylan are grass derived xylan just like the bagasse xylan, the bamboo has structural and molecular properties that are closely related to those of wood than grasses (Wilkie, 1979, Chapter 3). Hence, their adsorption behaviour of the bamboo xylan onto the cotton lint would be similar to that of wood xylan (Fig. 7.1d). The bamboo is an emerging feedstock in South Africa, as such, and could be an alternative source of xylan to *E. grandis* and *P. patula* feedstocks that can be modified for adsorption onto cellulosic materials during the pulp processing. However, as shown in Chapter 3, the low efficiency of extracting the xylan from the bamboo feedstocks might be a limiting factor for their use.

7.3.5 Effect of xylan degree of polymerisation and side chain substitution on adsorption onto cellulosic materials

Excessive decrease in degree of side chain substitution and polymerisation of extracted xylans may have negative impact on enzyme aided adsorption onto cellulosic materials. The adsorption of the H₂O₂ bleached bagasse and *E. grandis* xylan gel onto the cotton lint decreased by 6% and 31% respectively with the application of the AguA (Fig. 7.1d). Yet as shown in Figure 7.1d, the adsorption of xylans extracted from bagasse and *E. grandis*, which both had a DP > 10, increased by 29 and 334% respectively. The major difference between the structures of the H₂O₂ bleached bagasse xylan and the unbleached bagasse xylan was the degree of polymerization (DP), which were < 10 sugar units in the H₂O₂ bleached bagasse xylan (Chapter 3). On the other hand, the *E. grandis* xylan and the *E. grandis* xylan gel differed mainly in the degree of MeGlcA substitution, which was less than 3% in *E. grandis* xylan gel compared to approximately 16% in the xylan extracted from *E. grandis* (Chapter 3). The electrostatic repulsion forces, covalent and hydrogen bonding involved in binding of the xylan onto the cellulosic material are among other factors, attributed to high degree of polymerisation and reduced degree of substitution (Ebringerová and Heinze, 2000; Walker, 1965; Sjöström, 1993; Schönberg et al, 2001; Dahlman et al, 2003). However, it appears that the removal of the glucuronic side chains beyond the 3% substitution prevented the adsorption of the *E. grandis* xylan gel onto the cotton lint. Unlike the complete removal of acetyl groups that favour xylan adsorption, complete or excessive removal of MeGlcA side groups may not favour the xylan adsorption onto cellulosic materials. This effect could be because the MeGlcA groups introduce surface charges onto the cellulosic materials, which increase the attractive forces for adsorption (Danielsson, 2007). It is reported that beyond a critical minimum for degree of polymerisation and degree of side chain substitution, the attraction forces between the xylan polymers and the cellulosic surface are reduced (Kabel et al., 2007). For example, critical minimum sizes of xylan backbone chain necessary for xylan adsorption have been reported, which ranged from 7 to 15 DP of xylose units (Walker, 1965; Vinken et al., 1995; Westbye et al, 2006; Kabel et al, 2007; Ramírez et al., 2008). A higher degree of adsorption of Eucalyptus xylan onto pulp fibres than that of birch xylan was observed by Danielsson (2007), which was attributed to higher residual content of MeGlcA in the

Eucalyptus xylan than birch xylan after both xylan fractions were subjected to kraft cooking. Therefore, in order to achieve an efficient adsorption of xylan from *E. grandis* onto cellulosic materials aided by the AguA, the xylan should have degree of polymerisation of at least >10 sugar units and degree of MeGlcA substitution of at least greater than 3 but less than 12.8%.

7.3.6 Effect of enzyme xylan aided adsorption on the physical and structural properties of the cotton lint

The application of the AguA during adsorption of arabinoglucuronoxylan could be used for preparing cellulosic products with improved water holding capacity. The results showed that the cotton lint xylan specific weight gain was the highest for cotton lint treated with arabinoglucuronoxylans in the presence of AguA and a cocktail of the AguA and AbfB compared to cotton lint treated in the presence of the AbfB (Fig.7.2). For example, the increase in the xylan specific weight gain of the cotton lint treated with *P. patula* xylan in the presence of the AguA had the xylan specific weight gain of 3 times that of the cotton lint treated with the *P. patula* xylan in the presence of the AbfB (Fig. 7.2). The higher xylan specific weight gain for the cotton lint treated with arabinoglucuronoxylan in presence of the purified AguA rather than in the presence of the AbfB is associated with the higher content of arabinose side chains in the adsorbed arabinoglucuronoxylans treated with AguA than those treated with the AbfB. The arabinose side chains are known to be hydrophilic (Izydorczyk et al., 1991; Biliaderis, 1995; Sternemalm et al., 2008) such that adsorption of xylan with higher arabinose content would increase the water absorption capacity and distribution of the cotton lint. Therefore, cellulosic products with different water adsorbing capacities can be produced for different industrial applications. Henriksson and Gatenholm (2002) reported improved wetting and liquid adsorption properties of cellulosic materials modified by adsorption of xylan.

Furthermore, the application of the AbfB, AguA and the cocktail of the two enzymes during xylan adsorption can be used to introduce varying xylan–cellulose interactions different from those that occurred with xylan adsorbed onto the cotton lint in their absence. The solid state ^{13}C -(CP/MAS) NMR spectra of cotton lint treated with bagasse and *P. patula* xylan in the presence of AguA and AbfB displayed altered splitting patterns and decreased intensities for C-1, C-4 and C-6 carbon resonances

compared to the splitting patterns and intensity of the corresponding carbon resonances in the solid state ^{13}C -(CP/MAS) NMR spectra of the cotton lint treated with bagasse and *P. patula* but in the absence of the purified AguA and recombinant AbfB (Figs. 7.4c and d). Variation in the splitting patterns and intensity of the carbon signals were different for the spectra of the cotton lint treated with the xylan in the presence of recombinant AbfB and the purified AguA and the cocktail of these enzymes (Figs. 7.4a-g). Such effects could be associated with the variations in the amount and structure of xylan that adsorbed onto the cotton lint in the presence and absence of the enzymes, which might have changed the purity of the cellulose through xylan-cellulose interactions. Lahaye et al. (2003) indicated that the change in the chemical shifts and the sharpening of the C-1, C-4 and C-6 carbon signals may be associated with mobility of loosely organized and/or interacting the cellulose polymers. Furthermore, Wan et al. (2010) indicated that changes occur in the C-4 region due to changes in hemicelluloses content, which affects the cellulose fibril aggregate. However, Fardim and Durán (2004) indicated that the characteristics rather than the content of the xylan influenced the properties of the cellulosic material onto which xylan was adsorbed.

It is possible to obtain different xylan-cellulose interactions because the selective removal of the side chains from the xylan biopolymer results in different conformation that could have affected their alignment onto the cellulosic microfibrils differently. Linder et al. (2003^a) reported globular xylan particles were found on the surface of cellulosic material after xylan adsorption. In addition, Westbye et al. (2006) observed different surface morphology on the cellulose fibril surface was due to the differences in the amount and alignment of xylan nano particles that precipitated onto the cellulosic material. Furthermore, Winter et al. (2006) found that adsorption of xylan onto the cellulosic material resulted in reduction of crystallinity and content of $\text{I}\alpha$ cellulose. In view of the reported literature, the observed changes in the solid state ^{13}C -(CP/MAS) NMR spectra suggest that the surface properties and the crystallinity of the cellulose in the cotton lint might have been altered differently with the *in situ* purified AguA and recombinant AbfB modification and adsorption of the xylan extracted from the different sources. Therefore, the modification of the structural and surface properties as a result of the AbfB and AguA aided adsorption may have a significant influence on tailor made functional properties of the cotton lint.

7.3.7 Enzyme aided xylan adsorption in industrial set up

The adsorption behaviour of the arabinoglucuronoxylan and glucuronoxylan extracted from the South African feedstocks onto the cotton lint exhibited in this study is a reflection that the xylan fractions from the South African feedstock were extracted with structural features such as degree of polymerisation, degree of substitution and substitution pattern of the side chains suitable for use as speciality in cellulose surface modifications. The successful *in situ* enzyme selective modification and adsorption of the extracted xylans onto the cotton lint, presents the AbfB and AguA as a potential non-invasive, efficient method for targeted modification and enhancement of their adsorption onto cellulosic materials and for other uses. The solid state ^{13}C -(CP/MAS) NMR displayed signals for carbon resonances originating from glucose units of the cellulosic residues in the cotton lint treated with xylans in the presence of the recombinant AbfB and the AguA to be equal in magnitude with carbon resonances of glucose units of the cellulose residues of the untreated cotton lint spectra (Fig. 7.4a-h). These results suggest that the *in situ* enzymatic modification and adsorption of the xylans preserved the integrity of the structure of the cotton lint. However, the use of purified AguA and recombinant AbfB as bio-based catalysts for *in situ* modification and adsorption onto cellulosic surfaces of the xylans extracted from the South African feedstock can easily be integrated, with minimal alterations, in the conventional kraft pulping process (Fig. 7.5). The integration of pre-extraction of xylans under mild-alkali conditions (Chapter 3) and the *in situ* enzyme aided targeted modification and adsorption of the xylans onto the pulp fibres in the pulping processes (Fig. 7.5) is amenable for improving the functional properties of both the xylans and the cellulosic materials.

The *in situ*, simultaneous enzyme aided modification and adsorption of xylans adsorption onto cellulosic materials present an energy saving and less polluting strategy for modifying surface and strength properties of cellulosic fibres. The conditions under which the AbfB and AguA increased the adsorption of the xylans onto the cotton lint, which were pH between pH 4.8 and pH 5.0 at 40°C, suggest a possibility of incorporating the enzyme aided xylan *in situ* modification and adsorption at the wet end of fibre processing stages in the kraft pulp and paper making process. It is expected that at the wet end of the kraft pulp and paper making process,

it would be feasible to adjust the temperature and pH of the cellulosic fibres pulp to be within the acceptable range for optimal functioning of the enzymes ($< 50^{\circ}\text{C}$ and $< \text{pH } 6.0$, Chapter 4). Therefore, in comparison to the thermo-chemical assisted adsorption of birch xylan onto cotton linters that was performed at 150°C and $\text{pH } 10$ (Henriksson and Gatenholm, 2002) and on softwood kraft pulp performed at 100°C and $\text{pH } 10$ (Westbye et al., 2006). Such conditions are close to those of kraft pulping (Sjöström, 1993) as such, excessive degradation of both the xylans and the cellulosic material may occur that could reduce the technical and economic efficiency of the xylan adsorption process. Hence the enzymatic aided adsorption would be more energy efficient and less polluting to the environment.

In addition, the use of *E. grandis*, *P. patula* and bamboo as sources of xylans for *in situ* enzyme modification and adsorption of xylans onto cellulosic material would make the integration of enzyme modification with xylan pre-extraction within the pulping processes convenient because these feedstocks are the existing major feedstocks for production of pulp in South Africa. Thus, the *in situ* simultaneous enzyme aided xylan modification and adsorption can easily be integrated in the pulping process initially proposed by Westbye et al. (2006), which involved extraction of xylans prior to pulping and allowing the xylans to be re-introduced for adsorption at various stages between the pulping and paper making processes in the kraft pulping and paper making process. In such pulping process Saake et al. (2005) proposed xylan adsorption to be performed shortly before hand sheet formation. For that reason, the side chain removing enzymes have a new role in diversifying manufacturing of speciality cellulosic products for wider applications in other industries apart from the pulp and paper industry.

7.4 CONCLUSION

The AbfB and AguA increased adsorption of soluble xylans onto the cotton lint. The application of AguA was more effective in increasing adsorption of *P. patula*, bamboo and bagasse xylans onto the cotton lint than the recombinant AbfB and a cocktail of the recombinant AbfB and purified AguA. However, the purified AguA was the most effective side chain removing enzyme for enhancing adsorption of water soluble xylans onto cellulosic materials and the cocktail of the two enzymes was more effective in increasing adsorption of the arabinoglucuronoxylans than the application of the recombinant AbfB alone. The best source of xylans for *in situ* enzyme aided adsorption onto cellulosic materials was *E. grandis*. The purified AguA and recombinant AbfB aided adsorption of the xylans onto the cotton lint introduced and improved hydration and structural properties. Therefore, AbfB and AguA are both speciality bio-based catalysts for xylan modification that have a new a role of enhancing the assembly of water soluble xylans onto cellulosic materials to improve the physical and functional properties of cellulosic materials.

Chapter 8: *In situ* enzymatic aided formation of oat spelt xylan nanohydrogels and encapsulation and slow release of horse radish peroxidase

ABSTRACT

Insoluble oat spelt xylan hydrogels formed by selective removal of arabinose side chains from oat spelt xylan by recombinant α -L-arabinofuranosidase (AbfB) were characterized for particle size, surface charge (zeta potential), morphology stability and ability to encapsulate and slowly release horse radish peroxidase (HRP). The oat spelt xylan hydrogels had particle sizes ranging from 18 nm to > 10,000 nm. The oat spelt xylan nanohydrogels were spherical and exhibited a negative zeta potential of up to -19 mV. The xylan concentration significantly ($P < 0.05$) influenced both the particle size and zeta potential of the oat spelt xylan nanohydrogels whereas the recombinant AbfB hydrolysis time was significant on zeta potential. In addition, the oat spelt xylan nanohydrogels aggregated more at oat spelt xylan concentration higher than 1.5% (w/v) and recombinant AbfB hydrolysis time beyond 17 h. On the other hand, the polyethylene glycol 1000 (PEG 1000) plasticisers had no significant effect on the oat spelt xylan nanohydrogel aggregation, particle size and zeta potential. The HRP was successfully encapsulated both during and after formation of the oat spelt xylan nanohydrogels and was released in active form for over a period of 180 min. Thus, the recombinant AbfB presents a novel tool that can allow *in situ* transformation of water soluble xylan into functional nanohydrogels and encapsulation matrices for slow and targeted release of bioactive agents.

8.0 INTRODUCTION

Xylans have special gelling and film forming properties for production of nanohydrogels with wide use in industries including pharmaceutical, food and medical industries (Ebringerová and Heinze, 2000). Nanohydrogels are being developed among other uses, for use as controlled, slow and targeted delivery systems (Reis et al., 2006). The major challenge has been in finding suitable materials and methods for preparation of the nanohydrogels that are non-toxic and biocompatible (Reis et al., 2006). Among the natural sources, biodegradable nanohydrogels are often prepared from (1-4)-glycosidic-linked polysaccharides such as cellulose and starch that exhibit gelling properties when in solution (Ebringerová and Heinze, 2000; Dumitriu, 2002). Interest in the production of nanohydrogels from biodegradable and non-toxic such as the xylans is growing due to increased environmental and health concerns associated with use of petroleum based polymers coupled with advances in nanotechnology and particle engineering. Although xylans are (1-4)-glycosidic-linked polysaccharides; preference of cellulose and starch for preparation of biodegradable nanohydrogels is due to the higher molecular mass and relatively low degree or absence of side chain substitution, leading to lower water-solubility (Ebringerová and Heinze, 2000). However, utilisation of xylans for production of insoluble nanohydrogels has become necessary because starch and cellulose have other existing competing uses as food additives, fibre for paper and sugars for biofuels production. Additionally, xylans are available in abundance, accounting for 25-35% biomass dry weight in higher plants (Timell, 1967). However, the relatively high water solubility of the xylans is a limiting factor that needs to be addressed.

The principle behind formation of insoluble xylan nanohydrogels from water soluble xylans is the reduction in the degree of side chain substitution and the alteration of the side chain distribution pattern (Ebringerová and Heinze, 2000; Linder et al., 2003^{ab}; Kabel et al., 2007; Sternemalm et al., 2008). Depending on the source, xylans contain a backbone chain of 1,4- β -linked-D-xylose residues to which L-arabinose, D-MeGlcA, short oligosaccharide side chains, *O*-acetyl groups and ferulic and *p*-coumaric are attached (Timell, 1967; Wilkie, 1979). Removal of these side chains from water soluble xylans to form nanohydrogels is often achieved through processes

such as hydrothermal treatment, supercritical antisolvent precipitation, and manipulation of pH and ionic charges in coacervation process (Gabrielii et al., 2000; Garcia et al., 2001; Hans and Lowman, 2002; Linder et al., 2003^{ab}; Coviello et al., 2007; Haimer et al., 2008). Such physical and chemical methods are not efficient because of being non selective in modifying the xylans. In addition, the chemical and physical methods may not allow *in situ* formation and encapsulation of sensitive bioactive agents that have to be delivered in pure and untransformed form. For instance, the use of the chemical methods for production of xylan nanohydrogels, in particular, have a high probability of affecting the purity of the formed xylan nanohydrogels and the encapsulated product due to residual chemicals that could also be toxic to the encapsulated products. Similarly, *in situ* encapsulation of sensitive bioactive agents may not be possible for xylan nanohydrogel formed under supercritical conditions (150 bar) (Haimer et al., 2008) and with hydrothermal treatments (temperatures higher than 100°C) (Henriksson and Gatenholm, 2002, Linder et al., 2003^{ab}) because of the denaturing effect of such conditions to biological compounds.

The hydrogels prepared as slow release delivery systems of biological materials are required to protect the functional properties of the encapsulated bioactive agents (Coviello et al., 2007). The important features for efficient functioning of the polysaccharide hydrogels as encapsulation matrices of bioactive agents include swelling ability, gas or liquid permeability, size, surface charge, morphology, assembling behaviour and chemostability. The importance of these hydrogel functional properties have been described for specific industrial use in detail by a number of workers (Cole, 1992; Peppas, 1997; Silva et al., 1998; Peppas et al., 2000; Caruso et al., 2000; Hans and Lowman, 2002; Dumitriu, 2002; Kishida and Ikada, 2002; Domb et al, 2002; Lindblad, 2003; Vaghefi and Savitzky., 2005; Yi et al., 2005, Wang et al., 2006; Coviello et al., 2007; Hornig et al., 2009). The encapsulation and slow delivery systems developed so far have a high risk of getting degraded prematurely before delivering the encapsulated substance to the target place. The functional properties of hydrogels for use as encapsulation matrices are influenced by the properties of the raw materials and preparation methods. Xylan nanohydrogels are known to be suitable for sustained targeted release of encapsulated products because of being chemostable as such are known to be resistant to digestion in the human

stomach and intestines (Ebringerová, et al., 2002) but are hydrolysable by enzymes present in the human colon (Nagashima et al., 2008). A study by Silva et al. (2007) showed that xylan coated magnetite microparticles were protected from gastric dissolution when ingested orally. The observed effect was an improvement in the performance of medical scanning procedures such as magnetic resonance imaging (MRI) that require sustained presence of the magnetite in the body. Polysaccharides such as xylans form hydrogels of a chemically and physically crosslinked hydrophilic polymer network capable of imbibing large amounts of water or biological fluids (Coviello et al., 2007), a property that suits their use as encapsulation matrices for bioactive agents. The xylan-derived nanohydrogels have added advantages because they possess antiviral, anti-ulcer and antitumor activities, which are not found in other natural polymers such as cellulose and starch (Ebringerová and Heinze, 2000; Ebringerová, et al., 2002; Saitô, 2005; Ciprian et al., 2008). Therefore, xylan hydrogels have potential for direct application in the biomedical, pharmaceutical, agriculture, water treatment, nutraceutical, bioremediation, production of functional packaging, sanitary and textiles products. For this reason, the transformation of the water soluble such as those extracted from bagasse, bamboo, *Pinus patula* and *Eucalyptus grandis* (Chapter 3) into nanohydrogels would allow these xylans to be used in such novel applications.

Formation of stable spherical oat spelt xylan hydrogels in the presence of bioactive agents would be particularly relevant for the development of biocompatible encapsulation and slow release system for delivering bioactive agents that have strict dosage requirements. Other workers have shown that xylan nanohydrogels could be produced by chemical crosslinking of water soluble polymers with other functional groups or by polymerization of the water soluble monomers (Lindblad et al., 2001, Hans and Lowman, 2002, Nagashima et al, 2008). To stabilise the morphology of the xylan nanohydrogels, thus preventing self assembling tendency of the nanoparticles, complexation procedures (Akiyoshi et al., 1998) and addition of plasticisers such as polyethylene glycol 1000 (PEG 1000) (Garcia et al., 2001; Hans and Lowman, 2002) have been used. However, such crosslinking methods produce nanohydrogels that might be incompatible for direct use in pharmaceutical applications (Van Tomme et al. (2008) because of traces of residual chemicals. Therefore, selection of non-toxic

and biocompatible methods for production of xylan hydrogels that would allow wider applications of the xylan nanohydrogels is inevitable.

Biological methods, such as use of enzymes, offer non-invasive methods of preparing xylan nanohydrogels from polymeric xylans (Chapters 4-6). The enzymes are naturally occurring compounds as such would enable to prepare xylan nanohydrogels that are biocompatible and less toxic compared to those prepared by chemical methods. Berlanga-Reyes et al. (2009) reported preparation of hydrogels through oxidative cross linking of feurolylated arabinoxylans by laccase. Insoluble hydrogels were prepared from water soluble arabinoxylans and arabinoglucoronoxylans by selective removal of arabinose side chains by the recombinant α -L-arabinofuranosidase (AbfB) (EC3.2.1.55) and purified α -D-glucuronidase with polymeric xylan substrate specificity (Chapter 4 and 5). The purpose of the study was to evaluate the potential of using the recombinant AbfB for *in situ* precipitation of water soluble oat spelt xylan into nanohydrogels and encapsulation of bioactive agent for slow release. Horse radish peroxidase (HRP) was used as a model bioactive agent. In the study, the morphology (particle size and shape) and surface charge (zeta potential) of the formed oat spelt xylan hydrogels were analysed. In addition, the effect of using additives such as the polyethylene glycol 1000 (PEG 1000) during the production of the xylan hydrogels on the morphological and surface charge characteristics was assessed.

8.1 MATERIALS AND METHODS

8.1.1 Materials

The oat spelt xylan (X-0627) containing arabinose, glucose and xylose in a 10:15:75 ratio was purchased from Sigma. The recombinant AbfB with volumetric activity of 18.0 nkat mL⁻¹ against *p*-nitrophenyl arabinofuranoside (*p*NPA) was produced in house (Chapter 4). The horse radish peroxidase (HRP) (EC 1.11.1.7) (Type VI-A P-6782) with specific activity of 1000 U mg⁻¹ against 2,2'-Azino-bis(3-ethylbenzthiazoline-6-sulfonic Acid) (ABTS), # A-1888) was purchased from Sigma. The H₂O₂ for use in the HRP activity assay was prepared from a 30% (w/w) stock solution purchased from Merck. The polyethylene glycol (PEG) 1000 (product # 81190) plasticizer was purchased from Merck. The buffers were prepared from analytical grade chemicals according to Gomori (1955). Unless specified otherwise, the water used in the study was de-ionised water (dH₂O).

8.1.2 Production and characterization of oat spelt xylan hydrogels

Oat spelt xylan solutions were prepared according to De Wet et al. (2008). About 2.5 mL oat spelt xylan solutions (1% w/v) were placed in test tubes into which 1 mL of recombinant AbfB with volumetric activity of 18.0 nkat mL⁻¹ was added. The volume of the reaction mixture was adjusted to 5 mL by addition of 0.05 M citrate buffer pH 5.0. The reaction was performed at 40°C in a water bath for 16 h. A control sample consisting of 2.5 mL oat spelt xylan was incubated under similar conditions in the absence of the recombinant AbfB. Precipitation of the oat spelt xylan was assessed visually and by taking pictures under the microscope. Stability of the xylan hydrogels was assessed by measuring the mean particle size distribution in the reaction mixture after 30 min, 3 h and 7 days of terminating the hydrolysis of the xylan by the recombinant AbfB using Nano ZS90 Zetasizer (Malvern Instruments, 2004), which determines particle size by measuring Brownian motion of the particles in a sample using Dynamic Light Scattering (DLS) Principle. The method calculates the velocity of the particle in a liquid when an electrical field is applied. Given the particle velocity, the electrical field applied, viscosity and dielectric properties, the zeta potential can be calculated using an inbuilt Malvern software programme (Malvern Instruments, 2004).

8.1.3 Effect of process parameters on size and surface charge properties of oat spelt xylan nanohydrogel.

The effects on the xylan nanohydrogel size and surface charge (zeta potential) of PEG 1000 plasticizer and oat spelt xylan concentrations and the time taken to selectively hydrolyse the oat spelt xylan by the recombinant AbfB were evaluated using a standard three factor-two level cubic plus star surface central composite design (CCD). The design consisted of 16 runs of which 8 were cube points (n_c), 6 were star points (n_s) and 2 were a combination of centre points in the cube portion of the design and centre points in the star portion of the design (n_0) (Statistica, 2008). The experimental region covered xylan concentrations (X1) of $1\% \leq X1 \leq 3\%$, oat spelt xylan hydrolysis time (X2) of $7 \text{ h} \leq X2 \leq 24 \text{ h}$ and PEG plasticizer concentration (X3) of $15\% \leq X3 \leq 40\%$ (Table 8.1). The central points for xylan concentration (X1), oat spelt xylan hydrolysis time (X2) and PEG plasticizer concentration (X3) were set at 2%, 15.5 h and 27.5% respectively. The independent variables were coded using Equations 8.1-8.4.

$$X_1 = (C1-2)/1 \quad \dots\dots\dots (8.1)$$

$$X_2 = (C3-15.5)/8.5 \quad \dots\dots\dots (8.2)$$

$$X_3 = (C2-27.5)/12.5 \quad \dots\dots\dots (8.3)$$

Where:

- X and C = Coded and natural independent values respectively
- 1, 2 and 3 = Subscripts for xylan concentration hydrolysis time and plasticizer.
- α = Rotatability was calculated from Equation 8.4:

$$\alpha = (n_c)^{1/4} = 1.6818 \quad \dots\dots\dots (8.4)$$

Where

- n_c = Number of cube points in the design (i.e., points in the factorial portion of the design) (Statistica, 2008). The $\pm\alpha$ values for oat spelt xylan concentration in natural form were 0.32 and 3.68%, for plasticizer 6.48 and

48.52% and for time 1.2 and 29.8 h. The response surface plot was fitted with a second order polynomial (Equation 8.5)

$$Z = \beta_0 + \beta_1 X_1 + \beta_{11} X_1^2 + \beta_2 X_2 + \beta_{22} X_2^2 + \beta_3 X_3 + \beta_{33} X_3^2 + \varepsilon \dots\dots\dots (8.5)$$

Where:

- | | | |
|---|---|--|
| Z | = | Particle size (nm) or zeta potential (mV). |
| $\beta_0 + \beta_1 \dots\dots\dots \beta_n$ | = | Linear regression coefficients. |
| $\beta_{11} \dots\dots \beta_{nn}$ | = | Quadratic regression coefficients. |
| ε | = | Error |
| X_1, X_2, X_3 | = | Xylan concentration, recombinant AbfB hydrolysis duration (h) and PEG 1000 concentration respectively. |

The sizes of effects for xylan concentration, recombinant AbfB hydrolysis duration and PEG 1000 concentration and their interactions were determined by regression analysis. In addition, a desirability function was used to determine the optimal set point for the parameters that would give the smallest particle size and the most negative zeta potential.

8.1.4 Preparation and characterisation of oat spelt xylan nanohydrogels in CCD experiments

Oat spelt xylan solution (2.5 mL) and PEG 1000 plasticizer of specified volume and concentration according to the experimental design (Table 8.1) were mixed in a test tube to which 1.0 mL of recombinant AbfB (volumetric activity $\approx 18.0 \text{ nkat mL}^{-1}$) was added. The volume of the reaction mixture was adjusted to 5 mL with 0.1M citrate buffer (pH 5.0). The reaction mixture was incubated at 40°C on a shaker with a rotating speed of 20 r.p.m. The oat spelt xylan hydrogels to which PEG was added, were analysed for mean particle size and zeta potential using a Zetasizer (Nano ZS90) as previously described in Section 8.1.2. The zeta potential of the AbfB formed xylan hydrogels was measured using a Zetasizer (Nano ZS90) operating based on a combination of Electrophoresis and Laser Doppler Velocimetry techniques (sometimes called Laser Doppler Electrophoresis) (Malvern Instruments, 2004).

Table 8.1: Central composite design for evaluating effect of polyethylene glycol 1000 (PEG 1000) on particle size and zeta potential of α -L-arabinofuranosidase produced oat spelt xylan hydrogels.

CODED VARIABLES*				NATURAL VALUES			COMPOSITION OF REACTION MIXTURE					OUTPUT	
Sample Identification	X1	X2	X3	Xylan Concentration (% w/v)	Time (h)	PEG 1000 (% xylan wt)	Volume Xylan (uL)	Volume Citrate 6.0 (uL)	0.1M buffer pH	Volume PEG 1000 Plasticizer (uL)	AbfB Enzyme ** (uL)	Estimated Mean Particle size (nm)	Zeta Potential (mV)
XEP_1	-1	-1	-1	1.00	7.00	15.00	2500	1475		25	1000	81	-13.0
XEP_2	-1	-1	1	1.00	7.00	40.00	2500	1433		67	1000	21	-13.2
XEP_3	-1	1	-1	1.00	24.00	15.00	2500	1475		25	1000	103	-10.4
XEP_4	-1	1	1	1.00	24.00	40.00	2500	1433		67	1000	235	-19.1
XEP_5	1	-1	-1	3.00	7.00	15.00	2500	1425		75	1000	10000	-1.03
XEP_6	1	-1	1	3.00	7.00	40.00	2500	1300		200	1000	10000	-2.35
XEP_7	1	1	-1	3.00	24.00	15.00	2500	1425		75	1000	10000	-2.37
XEP_8	1	1	1	3.00	24.00	40.00	2500	1300		200	1000	10000	-9.16
XEP_9	-1.68179	0	0	0.32	15.50	27.50	2500	1485		15	1000	292	-15.5
XEP_10	1.68179	0	0	3.68	15.50	27.50	2500	1332		168	1000	10000	-9.5
XEP_11	0	-1.68179	0	2.00	1.20	27.50	2500	1408		92	1000	4378	-5.18
XEP_12	0	1.68179	0	2.00	29.80	27.50	2500	1408		92	1000	74	-8.41
XEP_13	0	0	-1.68179	2.00	15.50	6.48	2500	1478		22	1000	144	-17.1
XEP_14	0	0	1.68179	2.00	15.50	48.52	2500	1339		161	1000	126	-10.8
XEP_15	0	0	0	2.00	15.50	27.50	2500	1408		92	1000	114	-12
XEP_16	0	0	0	2.00	15.50	27.50	2500	1408		92	1000	114	-12

* Three factor standard two cubic plus star surface central composite design consisting of 16 runs, with the number of cube points (n_c) = 8, number of star points (n_s) = 6 and combined number of center points in the cube portion of the design and the number of center points in the star portion of the design (n_0) = 2,

** α -L- arabinofuranosidase (AbfB) with volumetric activity of about 18.00 nkat mL⁻¹.

The results were analysed using an inbuilt Malvern software programme (Malvern Instruments, 2004). The morphology of the particles in the mixture was assessed under a microscope to which a camera (Nikon Ellipse E400) was attached (magnification of X600).

8.1.5 Encapsulation and release of horse radish peroxidase in AbfB formed oat spelt xylan hydrogels

The oat spelt xylan and recombinant AbfB reaction mixture was prepared as described in Section 8.1.2 in two sets of test tubes. In one set, 50 and 100 μL horse radish peroxidase (HRP Type VI-A P6782, EC 1.11.1.7) with a protein concentration of 1 mg mL^{-1} and specific activity of 1000 U mg^{-1} against 2,2'-Azino-bis (3-ethylbenzthiazoline-6-sulfonic Acid) (ABTS), was added. The volume of the reaction mixtures were adjusted to 5 mL by adding 0.05 M citrate buffer (pH 5.0). The recombinant AbfB selective hydrolysis of the oat spelt xylan was performed either with or without HRP at 40°C for 24 h. The total volume for the reaction mixtures of both sets was 5 mL. The reactions were stopped by placing the test tubes in water containing ice. After 1 h of terminating the reactions, 50 and 100 μL of HRP supernatant were added to the second set. Subsequently, both sets were stored at 4°C for at least 24 h prior to testing for HRP positive encapsulation. Two control samples: (1) containing oat spelt xylan solution and the recombinant AbfB in the absence of HRP and (2) containing HRP and oat spelt xylan with HRP but without the recombinant AbfB, were processed under similar incubation conditions.

Samples of xylan precipitates with a volume of 2 mL were centrifuged at 10 000 rpm for 5 min at 4°C. The supernatant was decanted to recover the pellet (hydrogel). The pellet was washed by re-suspending it in one volume Milli-Q H_2O and again centrifuged at 10 000 rpm for 5 min at 4°C. The process was repeated until there was no trace of HRP activity in the decanted water. The HRP activity water was tested using a modified version of the HRP assay activity protocol described by Sigma. About 20 μL of the xylan hydrogel was placed in eppendorf in which 100 μL of 9.1 mM ABTS (prepared in 100 mM potassium phosphate buffer pH 5.0) and 100 mM potassium phosphate buffer pH in de-ionized water to make total volume of 265 μL was added. About 5 μL H_2O_2 (0.3%) prepared from 30% (w/w) stock solution

(Merck) were added to the mixture. Thereafter, the reaction mixture was incubated in the dark for 10 min. The assay was conducted at room temperature (25°C).

The degree of encapsulation and rate of release of the HRP from the oat spelt xylan hydrogels formed from selective hydrolysis of oat spelt xylan by the recombinant AbfB were determined for HRP added before and after AbfB xylan hydrogel formation. The degree of encapsulation was calculated based on the changes in the optical density measured at 405 nm (OD_{405nm}) in a microplate reader (xMark™ Bio-rad) against a reagent blank, which contained ABTS, buffer and H_2O_2 . The change in the OD_{405nm} due to oxidation of ATBS substrate by the HRP was read after 10, 30, 60, 120 and 180 min from the commencement of the HRP activity assay. The samples remained in the dark during the measurement intervals. The rate of diffusion of the HRP out of the oat spelt xylan hydrogel was defined as the rate of change of the OD (OD_{405nm}) per unit time ($\Delta OD \text{ min}^{-1}$). In the HRP activity assays, the ATBS oxidized by non-encapsulated HRP was used as a positive control whereas, a mixture of ATBS and the oat spelt xylan hydrogels without encapsulated HRP was used as a negative control.

8.2 RESULTS

8.2.1 Physical characteristics of recombinant AbfB formed oat spelt xylan hydrogels

The arabinose side chains of oat spelt xylan selectively hydrolysed by the recombinant AbfB resulted into formation of nanohydrogel precipitates that aggregated with time. The aggregation of the oat spelt xylan nanohydrogel resulted in a clear two phase solid- liquid separation (Fig. 8.1a). The xylan nanohydrogels formed by selective hydrolysis of water soluble oat spelt xylan by the recombinant AbfB appeared spherical in dispersed form (Fig. 8.1b). However, irregular shaped oat spelt xylan hydrogels precipitates prevailed with increased aggregation (Fig. 8.1b). The PEG 1000 plasticiser showed no significant effect ($P < 0.05$) on particle size and aggregation behaviour of the oat spelt xylan hydrogels (Figs. 8.1c and d). The hydrogels formed by the selective hydrolysis of oat spelt xylan by the recombinant AbfB were less aggregated at oat spelt xylan concentration of 0.32% (w/v) xylan (Fig. 1c) compared to oat spelt xylan hydrogels formed at xylan concentration of 3.68% (w/v) (Fig. 8. 1d).

In the absence of the PEG plasticiser, the particle size range of the oat spelt xylan hydrogels was estimated to be between were 30 min after terminating the AbfB hydrolysis were between 18 and 700 nm in diameter (Fig. 8. 2a). The bulk of the oat spelt xylan hydrogels attained a mean particle size within the 100 nm range during the measurement time interval (Fig. 8. 2a). However, after a 2.5 h interval (thus 3.0 h later), the particle size range of the oat spelt xylan hydrogels was between 28 nm and 1 280 nm with the bulk of the xylan hydrogels attaining sizes between 200 nm and 1 μ m (Fig. 8 2c). Storage of the oat spelt xylan hydrogels for a period of 7 days at 4°C resulted in an increase in the particle sizes and formation of hydrogels with irregular shapes. After the 7 days of storage, the particle size ranged from 78 nm to 1.48 μ m (Fig. 8. 2e). The particle size of the control samples (oat spelt xylan without the recombinant AbfB) was initially between 150 and 200 nm (Fig. 8. 2b) but after 7 days of storage the particle size range was from 100 nm to 1 μ m (1000 nm) with the bulk of the hydrogels attaining particle sizes of < 1000 nm (Fig. 8. 2f).

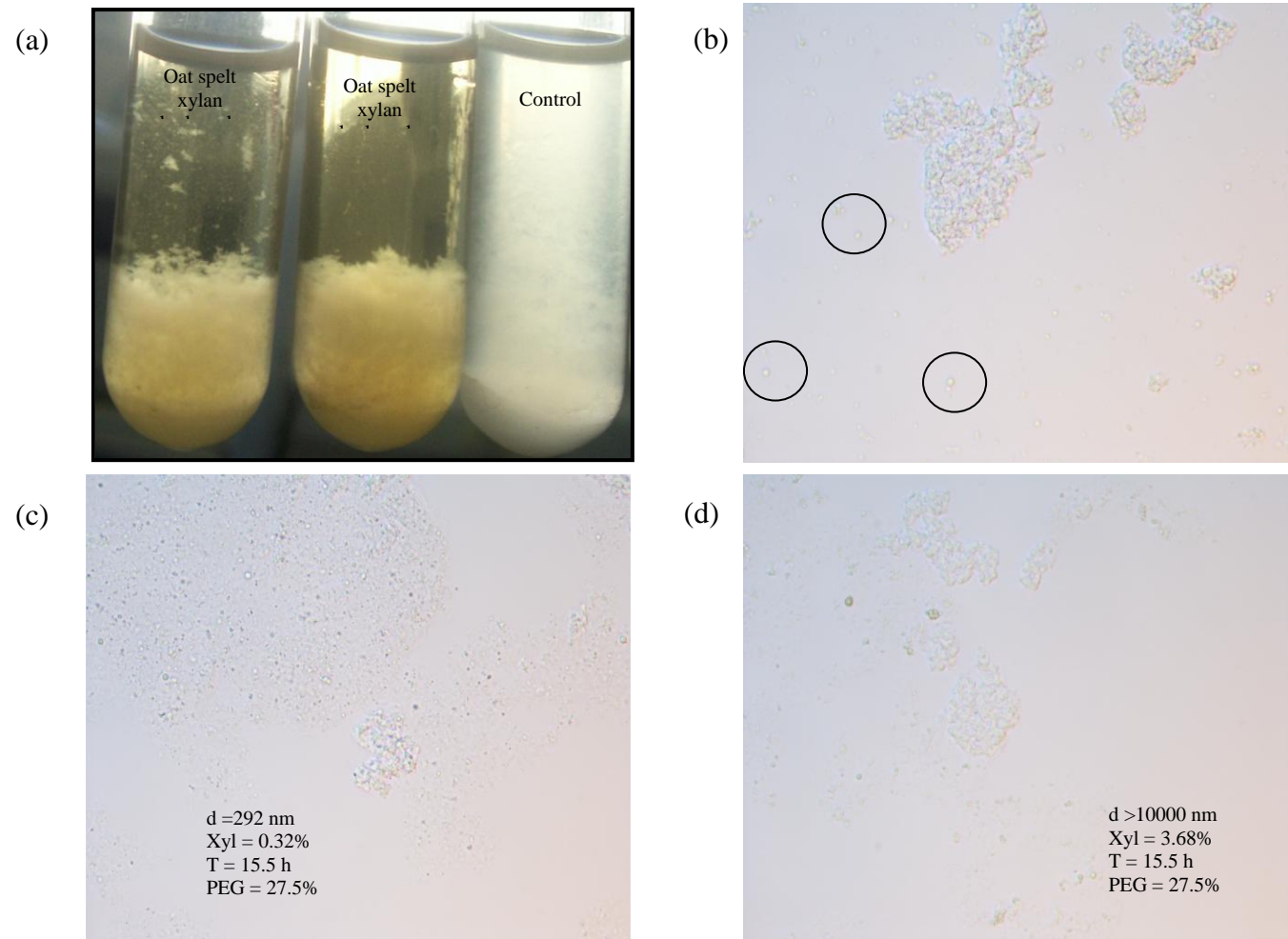


Figure 8.1: Physical characteristics of oat spelt xylan hydrogel formed upon selective hydrolysis of oat spelt xylan by the recombinant AbfB (a) resulting into precipitation and aggregation (two phase solid –liquid separation), (b) aggregation at xylan concentration = 2.0%, hydrolysis time = 15.5 h in absence of plasticiser (c) aggregation in presence of PEG 1000 plasticizer (PEG) = 27.5%, Xylan (Xyl) = 0.32% and hydrolysis time = 15.5 h) and (d) aggregation behaviour of in the presence of PEG 1000 plasticizer (PEG) = 27.5%, Xylan = 3.68% w/v, hydrolysis time = 15.5 h. Circles are showing some of the spherical particles in suspension. The d = diameter (nm).

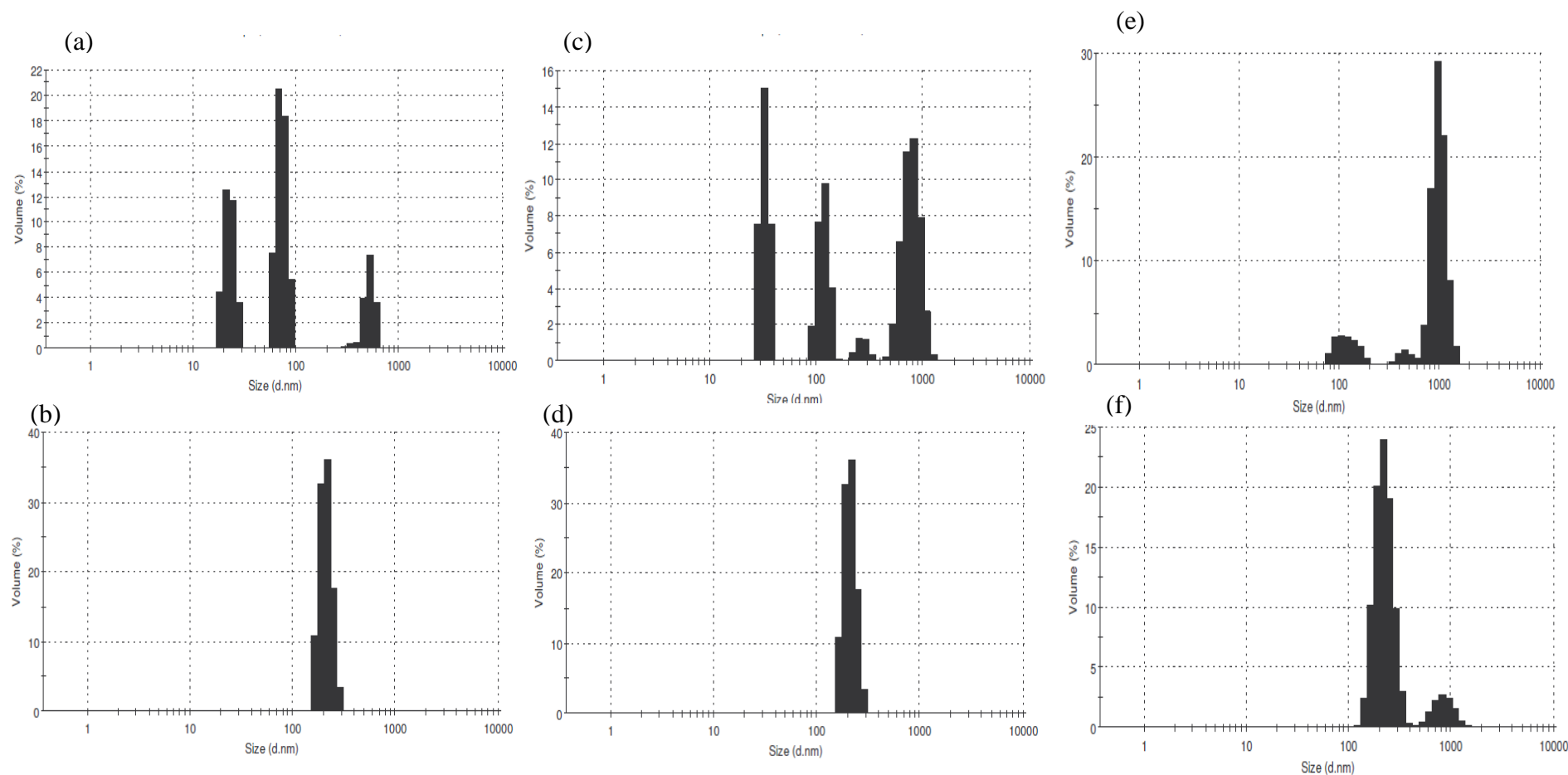


Figure 8.2: Particle size distribution by volume statistics of oat spelt xylan hydrogel formed from selective hydrolysis of oat spelt xylan by recombinant AbfB (a) measured after 30 min of terminating hydrolysis (b) unmodified oat spelt xylan measured after 30 min (c) oat spelt xylan hydrogel formed from selective hydrolysis of oat spelt xylan by recombinant AbfB measured after 3h of terminating hydrolysis (d) unmodified oat spelt xylan measured after 3 h (e) oat spelt xylan hydrogel formed from selective hydrolysis of oat spelt xylan by recombinant AbfB measured after 7 days of terminating hydrolysis (f) unmodified xylan measured after 7 days.

8.2.2 Dependency of size and surface charge properties of oat spelt nanohydrogels on xylan concentration, enzyme hydrolysis time and plasticizer concentration.

The effects of xylan concentration, recombinant AbfB hydrolysis time and PEG 1000 concentration on particle size and zeta potential of nanohydrogels were determined from response surface plots shown in Figures 8.3a and b. The response surface plotted at constant recombinant AbfB hydrolysis time of 15.5 h showed particle sizes of the oat spelt xylan nanohydrogels of up to 20000 nm (20 μ m) at concentration of PEG 1000 of 27.7% and 3% of xylan (Figs. 8.3a i & ii). The response surface plot for the particle size fitted the polynomial quadratic equation with regression coefficient (R^2) of 0.88 (adjusted $R^2 = 0.81$) after ignoring linear and quadratic effects of the interaction between the PEG 1000 concentration, oat spelt xylan concentration and enzyme hydrolysis time that were not significant ($P < 0.05$). The surface plot for zeta potential showed a downward curvature with time but a linear relationship with PEG 1000 plasticiser and xylan concentrations (Figs. 8.3b i-iii).

The oat spelt xylan hydrogels produced from the selective hydrolysis of the oat spelt xylan by the recombinant AbfB gave negative zeta potential values ranging from -19 to -1.03 mV (Table 8.1). A zeta potential value of -14.3 mV was estimated for oat spelt xylan hydrogels formed at oat spelt xylan concentration of 1.5%, PEG 1000 concentration of 27.5% and recombinant AbfB hydrolysis time of 15.5 h, whereas, at xylan concentration of 2% gave the zeta potential of the oat spelt xylan hydrogels was -12.5 mV (Fig. 8.3b ii). At zero recombinant AbfB xylan hydrolysis time, PEG 1000 concentration of 27.5% and xylan concentration of 2%, the zeta potential of the oat spelt xylan was estimated to be -2 mV, whereas, recombinant AbfB hydrolysis time of 1 h the zeta potential of the oat spelt xylan hydrogels was estimated to be -3.32 mV (Fig. 8.3b iii). The zeta potential value of the oat spelt xylan hydrogels was -9.0 mV when the recombinant AbfB hydrolysis time was increased to 28 h (Fig. 8.3b iii). The response surface plot for the effect of the PEG plasticizer concentration, oat spelt xylan concentration and recombinant AbfB hydrolysis time on zeta potential fitted the polynomial quadratic equation with R^2 of 0.74 and adjusted $R^2 = 0.56$ after ignoring insignificant interaction effects ($P < 0.05$) of the independent variables.

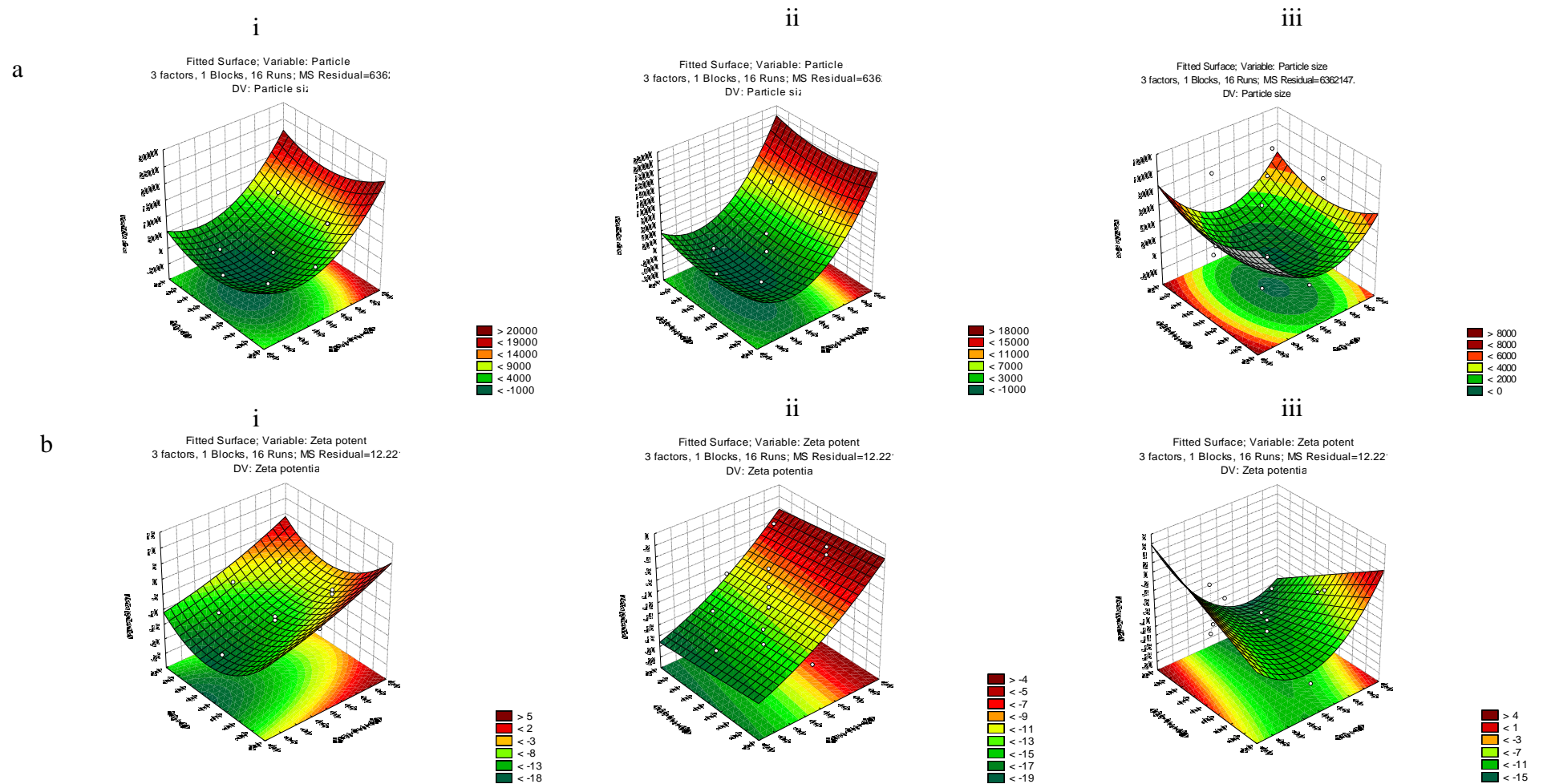


Figure 8.3: Response surface plots showing effect on (a) particle size and (b) zeta potential of (i) AbfB hydrolysis time and xylan concentration at 27.5% PEG 1000 (ii) PEG 1000 concentration and xylan concentration at 15.5 h and (iii) PEG1000 and time at xylan concentration of 2%. The response surface plots for particle size fitted the quadratic model with $R^2 = 0.88$ and adjusted: $R^2 = 0.81$ and for Zeta potential the surface plot fitted with $R^2 = 0.74$ and adjusted $R^2 = 0.56$. Significance of the effects was calculated $p < 0.05$.

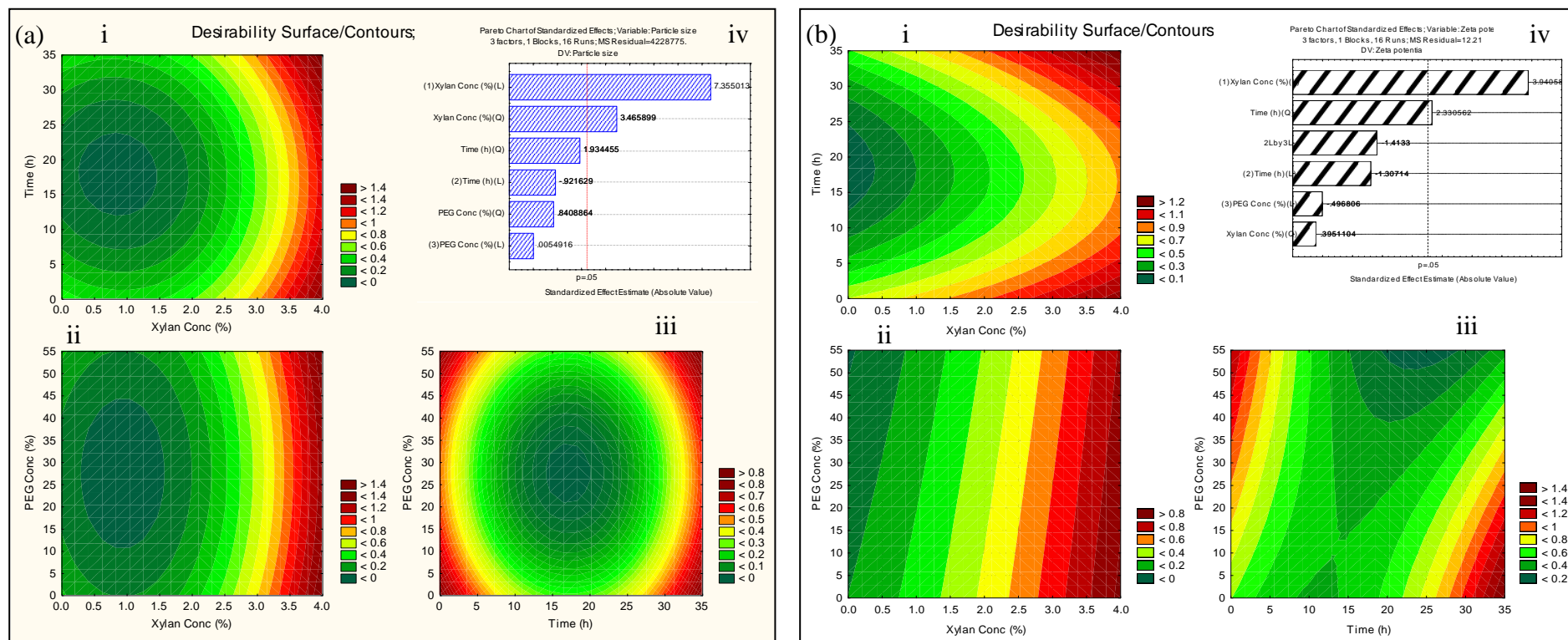


Figure 8.4: Desirability surface plot of the effects of xylan concentration, PEG 1000 and AbfB hydrolysis duration on (a) particle size and (b) zeta potential. Plot (i) shows interaction between time and xylan concentration; (ii) PEG 1000 concentration and xylan concentration, (iii) PEG 1000 and time (iv) Pareto charts showing size and significance of the main effects and interactions of the independent variables. In Pareto charts, the size of the bar charts depicts the size of effect of the independent variables. The vertical dotted line denotes $P < 0.05$ significant level. The model fitted the particle size response surface plots with $R^2 = 0.88$; Adjusted $R^2 = 0.81$, MS Residual = 4228775. The model fitted the Zeta potential response surface plots with $R^2 = 0.74$; Adjusted $R^2 = 0.56$ and MS Residual = 49.2943.

The oat spelt xylan hydrogels formed from selective hydrolysis of the oat spelt xylan by the recombinant AbfB had the smallest particle size at xylan concentration of 1%, PEG concentration of 25% and hydrolysis time of 17.5 h (Figs. 8.4a i-iii). The oat spelt xylan concentration showed significant linear and quadratic effects ($p < 0.05$) on particle size of the xylan hydrogels (Fig. 8.4a iv). The largest negative zeta potential values were obtained at a xylan concentration of $< 1\%$ (Fig. 8.4b i & ii) and at hydrolysis time of > 15 h (Fig. 8.4b iii). In addition, the xylan concentration and oat spelt xylan hydrolysis time had significant linear and quadratic effects on zeta potential ($p < 0.05$) respectively (Fig. 8.4b iv).

8.2.3 Encapsulation of HRP in oat spelt xylan nanohydrogels

After rinsing of the oat spelt xylan nanohydrogels encapsulating the HRP with Milli-Q H₂O (thus rinsing until HRP activity against ABTS substrate was no longer detectable in the waste water), the HRP activity was detectable in the oat spelt xylan nanohydrogels (Fig. 8.5a). The residual activity of the HRP in the oat spelt xylan hydrogels was reflected by optical density at 405 nm (OD_{405nm}). The OD_{405nm} was less when the HRP was added before than after formation of oat spelt xylan nanohydrogels (Fig. 8.5b). At HRP dosage level of 100 μ L, the ratio of the OD_{405nm} of the HRP added before formation of the oat spelt xylan nanohydrogels to that of the HRP added after the formation of the oat spelt xylan hydrogels and to that of HRP in control xylan (in the absence of the recombinant AbfB) was 1.0:11.0:5.1 (Fig. 8.5b). A similar trend was observed at HRP dosage level of 50 μ L whereby the ratio of the OD_{405nm} was 1: 6.5:1.7 respectively (Fig. 8.5b).

8.2.4 Release characteristics of HRP from oat spelt xylan nanohydrogels

The release of the HRP from oat spelt xylan nanohydrogels were monitored by change of the OD_{405nm} for a period of 180 min (3 h). After 10 min of terminating the encapsulation process, the OD_{405nm} of the HRP that was added before and after formation of the oat spelt xylan nanohydrogels were 1.276 and 0.882 respectively (Fig. 8.5c). The OD_{405nm} of HRP encapsulated during (HRP added before) and after formation of the oat spelt xylan nanohydrogels further increased to OD_{405nm} 3.74 and 5.0 after 180 min respectively (Fig. 8.5c). The OD_{405nm} for the activity of non-entrapped HRP monitored over the same period remained relatively steady at

approximately OD_{405nm} of 11 before dropping to 10.0 after 180 min (Fig. 8.5c). The rate at which the HRP was released from the oat spelt xylan matrix (defined as the change in OD_{405 nm} per unit time (U min⁻¹) was 0.07 U min⁻¹ in the initial 20 min for the HRP encapsulated before formation of oat spelt xylan nanohydrogels and 0.1 Umin⁻¹ for HRP encapsulated after AbfB hydrolysis of the oat spelt xylan (Fig. 8.5c). Over a 50 min measurement time interval, the rate of release of the HRP encapsulated during the AbfB hydrolysis of the oat spelt xylan was 0.01 U min⁻¹ whereas, for HRP encapsulated after the formation of oat spelt xylan hydrogels was 0.012 U min⁻¹. Beyond the 50 min interval, the release rate of the HRP encapsulated after formation of oat spelt xylan nanohydrogels stabilized at 0.013 U min⁻¹, whereas, the rate of release of the HRP encapsulated before the AbfB hydrolysis of oat spelt xylan continued to decline by 50% for the successive measurement time intervals 110 and 170 min (Fig. 8.5c).

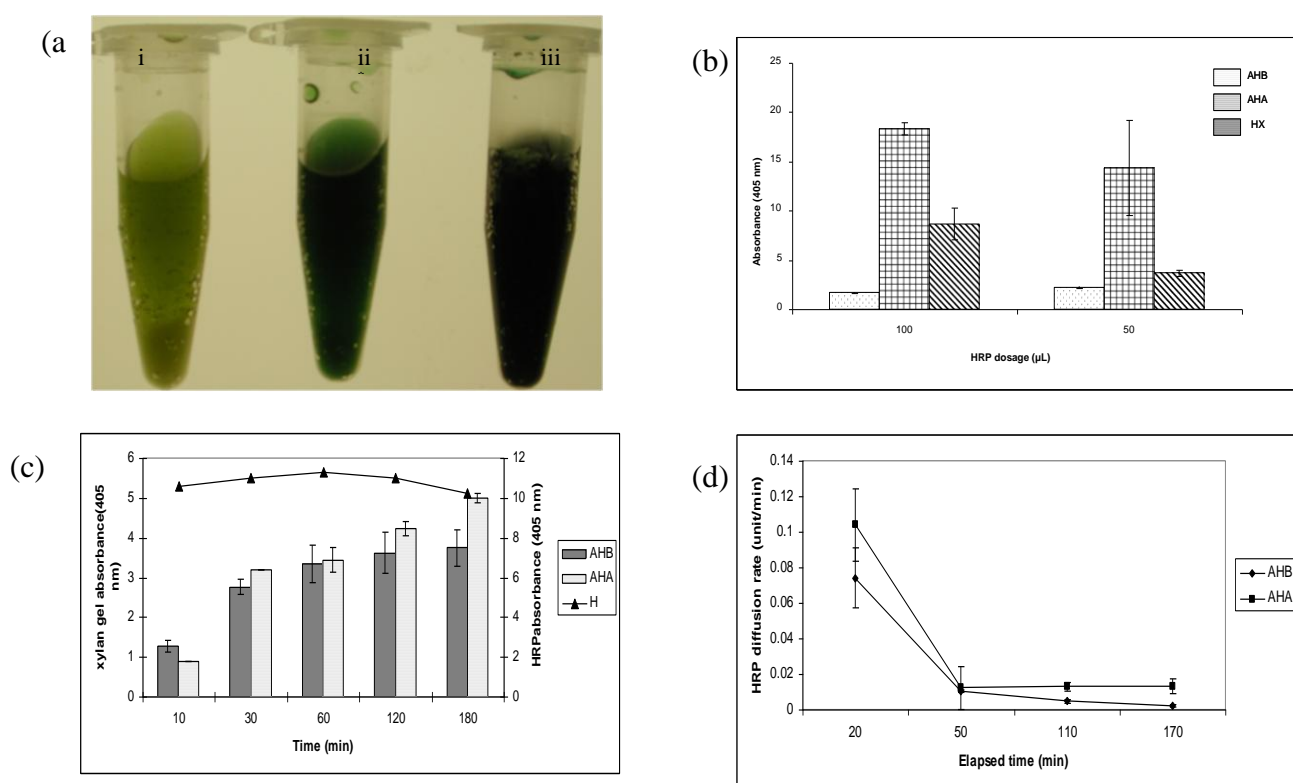


Figure 8.5: HRP release from (a) (i) -ve control, thus from oat spelt xylan hydrogel without HRP, (ii) oat spelt xylan hydrogel with HRP (iii) HRP +ve control, (b) residual activity in oat spelt reaction mixture in which HRP was added before (AHB) and after (AHA) in AbfB hydrolysis of the oat spelt xylan at dosage levels of 50 and 100 µL and of HRP in un hydrolysed oat spelt xylan (HX), (c) progression of diffusion of HRP out of oat spelt xylan gel for HRP added before AbfB oat speltxylan hydrolysis (AHB) and after AbfB oat spelt xylan hydrolysis (AHA). The line graph represent stability of HRP +ve control (H) during the monitoring period, and (d) the rate of diffusion for HRP out of the AbfB formed oat spelt xylan gel with HRP added before formation of xylan hydrogels (AHB) and after formation of xylan hydrogels (AHA) . The HX denotes residual activity of HRP in untreated xylan.

8.3 DISCUSSION

8.3.1 Characteristics of hydrogels produced by α -L-arabinofuranosidase (AbfB) hydrolysis of oat spelt xylan

The xylan hydrogels prepared by selective hydrolysis of the oat spelt xylan by the recombinant AbfB had particle sizes as small as 18 nm (Fig. 8.1c). Such nanohydrogels were smaller than the smallest nanoparticles (162 nm) formed by chemical methods such as esterification methods (Daus and Heinze, 2009) and nanohydrogels (371 nm) prepared from corn cob xylan by coacervation method (Garcia et al., 2001). Therefore, the xylan nanohydrogels formed from hydrolysis of the oat spelt xylan by the recombinant AbfB would enable their use as nanoencapsulation matrices of bioactive substances for slow release in smaller uniform doses compared to the larger nanohydrogels reported by Daus and Heinze (2009) and by Garcia et al. (2001). The formation of xylan nanohydrogels a result of selective removal of arabinose side groups from the oat spelt xylan by the recombinant AbfB was also evident from the selective hydrolysis of xylan extracted from bagasse and bamboo feedstocks found in South Africa and of birch xylan in selective hydrolysis by the purified α -D-glucuronidase (AguA) (Chapters 5-6). Hence, the recombinant AbfB and the purified AguA presents new ways of preparing nanohydrogels from water soluble xylan as alternative to the complex and costly methods such as esterification, multiple emulsion, chemical precipitation, polyelectrolyte complexation and desolvation (Garcia et al., 2001; Wang et al. 2006; Silva et al., 2007; Daus and Heinze, 2009), and hydrothermal chemical treatments (Linder et al., 2003^{ab})

The nanohydrogels prepared from selective hydrolysis of oat spelt xylan were spherical in shape. However, the particle size increased and the morphology of the oat spelt xylan nanohydrogels became irregular with time due to aggregation (8.1a-d). As a result, the oat spelt xylan nanohydrogel suspension, with time, contained a mixture of spherical but irregularly shaped xylan nanohydrogels and microhydrogels (Fig. 8.2a). The results showed that the sizes of the oat spelt xylan nanohydrogels measured between 18-700 nm after 30 min of terminating the hydrolysis for the selective hydrolysis of the oat spelt xylan by the recombinant AbfB (Fig. 8.2a) but after 3 h, the

sizes of the oat spelt xylan nanohydrogels increased further (Fig. 8.2c) and attained a size range of 78 to 1480 nm after 7 days (Fig. 8.2e). The tendency of the nanohydrogels to aggregate and increase in size with time was earlier reported by Linder et al. (2003^a). The irregular shaped and heterogeneous nanohydrogels formed (Fig. 8.1a) as a result of the self aggregation behaviour might not be desirable for encapsulation and delivery of bioactive agents that may be required in specific constant dosages (Kumar, 2000). Therefore, xylan nanohydrogels formation during the selective hydrolysis of the oat spelt xylan by the recombinant AbfB may need to be engineered in order to prevent or reduce the aggregation behaviour. The tendency of the xylan hydrogels to aggregate is attributed to reduced electrostatic repulsion forces between the xylan nanohydrogels (Muller et al., 2001; Kabel et al., 2007; Hornig and Heinze, 2008). Furthermore, Linder et al. (2003^a) indicated that such aggregation is facilitated by presence of small amounts of aromatic substituents covalently bonded to lignin residues. The ¹³C NMR spectra of the oat spelt xylan showed the presence of a carbonyl (COO-) functional group, which might originate from such compounds (Chapter 3). According to Hornig and Heinze (2008), the hydrogel aggregation can be prevented by creating a balance between the hydrophilic and hydrophobic parts of the xylan polymer. Other workers have shown that the morphology of the hydrogels is stabilised by addition of additives such as plasticisers, during particle formation (Garcia et al., 2001). Possibly, the aggregation of the nanohydrogels could be prevented by continuous harvesting of the xylan nanohydrogels at their rate of formation. Alternatively, methods such as sonication could be employed in the downstream processes to ensure there is continuous dispersion of the xylan hydrogels. The methods for preventing the self aggregation behaviour of the xylan hydrogels need to be investigated.

8.3.2 Effect of oat spelt xylan hydrogels of xylan and PEG1000 concentrations and hydrolysis time on particle size and zeta potential

The mean particle sizes of the oat spelt xylan hydrogels were significantly affected ($p < 0.05$) by the oat spelt xylan concentration but not by the hydrolysis time and PEG1000 concentration (Fig. 8.4a iv). The selective hydrolysis of the oat spelt xylan by the recombinant AbfB resulted in formation of hydrogels with mean particle size of 292 nm at oat spelt xylan concentration of 0.32% (w/v) whereas, at oat spelt xylan concentration of 3.68% (w/v) the xylan hydrogel mean particle size was ≥ 1000 nm

(Table 8.1). The results suggest that xylan nanohydrogels with tailor made particle sizes could be engineered during selective hydrolysis of xylan by controlling the xylan concentrations. The differences in the sizes of the xylan hydrogels at the two xylan concentrations can be visually seen in Figures 8c and 8d respectively. The tendency to produce xylan microhydrogels rather than nanohydrogels at higher xylan concentrations is in agreement with the findings of Garcia et al. (2001) who reported formation of xylan microhydrogels instead of nanohydrogels with xylan concentration of higher than 100 mg mL^{-1} (10% w/v). The response contour plots (Fig. 8.4a (i-iii)) showed that oat spelt xylan hydrogels of nano sizes of less than 100 nm could be optimally obtained with recombinant AbfB selective hydrolysis of oat spelt xylan at xylan concentrations ranging from 0.5 to 1.5% for 15 to 20 h (Fig. 8.4a i-iii).

The xylan concentration at which the selective hydrolysis of the oat spelt xylan by the recombinant AbfB did not result in formation of oat spelt xylan hydrogels of nano-scale size was less than the xylan concentration reported by Garcia et al. (2001) for preparation of hydrogels from corn cob xylan using chemical method. At higher xylan concentration, the hydrogels are inherently forced into self assembling behaviour through inter and intra polymer bonding, which could lead to formation of bigger but irregular shaped hydrogels compared to hydrogels formed at lower xylan concentration. However, because of their shear thinning and thixotropic characteristic (Ebringerová and Heinze, 2000) the aggregation of the arabinoglucuronoxylans such as those extracted from corn cob (Garcia et al., 2001) may be more prevalent at a higher xylan concentrations compared to arabinoxylans, which have shear thinning but without the thixotropic properties (Ebringerová and Heinze, 2000). This phenomenon could probably be due to lack of acid groups in arabinoxylans.

It is also possible that the oat spelt xylan concentration beyond 3% used in this study exceeded the substrate saturation level of the recombinant AbfB, which might have resulted in getting a mixture of modified and un modified xylan and therefore having varying magnitudes of attractive and repulsion forces within and between them. The presence of the varying attractive and repulsion forces might have led to the irregular conformations and sizes of the xylan nanohydrogels. The ability of the xylan polymers to attain varying conformation when in solution is a well known phenomenon, which is influenced partly by electrostatic forces (Linder et al., 2003^a;

Ebringerová et al., 2005). However, Linder et al. (2003) and Kabel et al. (2007) indicated that varying degrees of aggregation behaviour of xylan are mainly as a result of the degree of change in the chemical structures of the xylan. Formation of uniform xylan hydrogels by enzymatic methods could possibly be addressed by considering the saturation kinetics of the enzymes.

The zeta potential for the xylan nanohydrogels was significantly affected ($p < 0.05$) by both the xylan concentration and recombinant AbfB hydrolysis time (Fig. 8.4b iv). The xylan nanohydrogels that were formed at high xylan concentrations resulted in less negative zeta potential values (Fig. 8.3a i-iii). For example, at xylan concentrations of 0.32% and 3.68% the zeta potential values were -15.5 and -9.5 mV respectively (Table 8.1). The higher zeta potential values (thus, less negative zeta potential values) are an indication of reduced electrostatic repulsion forces on the surface of the xylan nanohydrogels. The electrostatic repulsion forces are responsible for keeping the particles in separation, which reduces the tendency of the hydrogels to aggregate (Muller et al., 2001; Hornig and Heinze, 2008). Therefore, the zeta potential values of at least -30 to -20 mV may lead to formation of hydrogels with stable physical structure (morphology) due to increased electrostatic repulsion (Muller et al., 2001; Hornig and Heinze, 2008). In this study, the zeta potential for the xylan hydrogels ranged from -19 to -1.0 mV (Table 1) indicating that it was one mV unit outside the stable range, hence, the observed aggregation behaviour resulting in formation of irregularly shaped oat spelt xylan microhydrogels aggregates (Figs. 8c and d).

The less negative zeta potential exhibited at longer recombinant AbfB hydrolysis time (>15.5 h) was possibly because of aging of the xylan nanohydrogels. Consequently, the electrostatic repulsion forces between the xylan hydrogels were reduced. Such behaviour could in part explain the self aggregation behaviour of the xylan hydrogels that prevailed with time (Fig. 8.2). Loss of gel firmness of 43% was reported in wheat arabinoxylan hydrogels formed by oxidation methods and was attributed to changes in chain structure (Carvajal-Millan et al., 2005). The results in Table 8.1 showed that hydrolysing the oat spelt xylan by AbfB for 15.5 h resulted into a more negative zeta potential value of -17.7 mV, which accordingly, provided more stable xylan nanohydrogels and less aggregation compared to oat spelt xylan nanohydrogels

formed by selective hydrolysis of the oat spelt xylan at hydrolysis time of 24 h (Table 8.1). However, the quadratic model fitting the response surface plot of the zeta potential as a function of xylan concentration, oat spelt xylan hydrolysis time and plasticiser concentration gave the lowest zeta potential of <-19 mV at recombinant AbfB reaction time ranging from 12 to 24 h (Figs. 8.3a i-iii).

The desirability analysis of the experimental data (Figs. 4b i-iii) showed that the optimal xylan concentration for obtaining the lowest zeta potential value could not be established in the region of the current experimental set-up possibly because such optimal region was not covered. This may explain the lower regression coefficients (R^2) of 0.74 (adjusted R^2 of 0.56) obtained from fitting the response surface quadratic model (Equation 8.4) to the response surface plots of zeta potential as a function of xylan concentration hydrolysis time and plasticiser (Figs. 8.3b i-iii). In contrast, a higher regression coefficient (R^2) of 0.88 (adjusted R^2 of 0.81) was obtained by fitting the quadratic model (Equation 8.3) to the surface plots of the experimental data for particle size as a function of hydrolysis time, plasticiser and xylan concentration that covered the optimal region (Figs. 8.3a i-iii).

In contrast to xylan concentration and AbfB hydrolysis time, there was no significant effect of PEG1000 plasticiser dosage on particle size and zeta potential of the AbfB formed oat spelt xylan nanohydrogels (Figs. 8.4a iv and 8.4b iv). The results are in contradiction with Garcia et al. (2001) who found the PEG 1000 plasticiser to have significant effect on both particle size and zeta potential of the xylan hydrogels using chemical methods. However, the PEG1000 plasticiser is known to be a hydrophilic, non-ionic polymer, which through covalent bonding or surface adsorption on the nanohydrogels would increase stability of the xylan nanohydrogels (Kumar, 2000; Garcia et al., 2001; Hans and Lowman, 2002). It is possible that the effect of the PEG 1000 plasticiser is specific to xylan type or method of preparing the xylan hydrogels. Furthermore, Garcia et al. (2001) showed that the effect of the plasticizers was enhanced by variation of pH or ionic strength that is prominent in chemical methods in particular the desolvation by coacervation method.

8.3.3 Horse radish peroxidase encapsulation and release

Horse radish peroxidase (HRP) was encapsulated and released in active form from the oat spelt xylan nanohydrogels formed by selective hydrolysis of the oat spelt xylan by the recombinant AbfB (Fig. 8.5a). The successful encapsulation of the HRP in oat spelt xylan nanohydrogels suggests that encapsulation of the bioactive agent could also occur in nanohydrogels formed by selective hydrolysis of xylan extracted from bagasse and bamboo by the recombinant AbfB (Chapter 5). The results show that positive encapsulation of the HRP occurred both during and after formation of the oat spelt xylan nanohydrogels (Fig. 8.5c). The ability to encapsulate the HRP both during and after xylan precipitation presents a more flexible and novel technology for encapsulation of both sensitive and none sensitive bioactive agents compared to the chemical methods such as those described by Gabriellii et al. (2000), Garcia et al. (2001), Hans and Lowman (2002) and Coviello et al. (2007).

Furthermore, the xylan nanohydrogels released the encapsulated HRP in its active form shown by the oxidation of ABTS for over a period of 180 min (Fig. 8.5c). The sustained release of the HRP over such period is a positive indication that the xylan nanohydrogels can be used as encapsulation matrix for targeted release of bioactive substances as well as non active compounds. The initial rate of release of the HRP encapsulated after xylan hydrogel formation was almost 1.5 times higher than the rate of release of the HRP encapsulated before formation of the hydrogel (Fig. 8.5d). However, the rate of release of the HRP encapsulated after formation of the xylan hydrogel stabilized after the initial outburst in the first hour, in contrast to the rate of release of HRP encapsulated before the formation of the hydrogel, which continued to decline with time (Fig. 8.5d). The phenomenon implies that the HRP encapsulated after the hydrogel formation was released with less restriction from the xylan hydrogel matrix than the one encapsulated before formation of the hydrogel. Such characteristic release shows that different encapsulation methods could be used for customised release. It is possible that the HRP encapsulated before the formation of the oat spelt xylan nanohydrogel was physically suspended inside the hydrogel matrix during the xylan hydrogel formation. Consequently, the release of the HRP would be restricted with the thickness of the xylan nanohydrogels matrix and crosslink ages formed between the polymers in the matrix.

Although both encapsulation of the HRP in the xylan nanohydrogels before and after their formation demonstrated the functionality of the xylan nanohydrogels as slow release delivery systems (Fig. 8.5d), the release of the encapsulated HRP from the xylan nanohydrogels initially showed an initial outburst (Figure 8.5d), which could be an indication of the presence of HRP that were loosely attached to the matrix of the xylan nanohydrogel. Such encapsulation behaviour is characteristic of higher loading of the bioactive agents in the nanoparticles (Hans and Lowman, 2002), which might not present an efficient encapsulation process. Therefore, the HRP dosage used in this study for the encapsulation in the xylan nanohydrogels might have been higher than what would have been optimally encapsulated. Hans and Lowman, (2002) indicated that an initial outburst release and longer sustained release is characteristic release of encapsulated bioactive agents from larger particles. However, the continued decline of the rate of release of the HRP without reaching a steady state in this study may be associated with the unstable physical conformations of the hydrogels due to aggregation noted in Figure 8. 1b. The release of the HRP that is adsorbed on the surface of the xylan nanohydrogels would be less affected by the changes in the morphology of the hydrogels than the HRP encapsulated inside the hydrogel because new movement restrictions may emerge with time in the diffusion pathway for the HRP encapsulated inside. Hence further investigation is required to develop ways of preventing aggregation of the xylan nanohydrogels upon their formation to ensure efficient encapsulation and targeted slow delivery of the encapsulated products.

8.4 CONCLUSION

The recombinant AbfB with polymeric xylan substrate specificity selectively hydrolysed soluble oat spelt xylan to form nanohydrogels with particle sizes as small as 18 nm. The xylan nanohydrogels formed were spherical and exhibited negative zeta potential of up to -19 mV. Stability and the morphological characteristics of the xylan nanohydrogels were influenced by the oat spelt xylan concentration and recombinant AbfB hydrolysis time. The xylan nanohydrogels self-assembled with higher xylan concentration and longer residence time during reaction and storage. In addition, the xylan nanohydrogels successfully encapsulated and sustained the release of the HRP for over 180 min. The characteristic and rate of release of the encapsulated HRP depended on the mode of encapsulation, hence there is potential for customised release of the HRP. Thus, the recombinant AbfB has presented a novel method for *in situ* transforming of water soluble xylan into functional nanohydrogels and nanoencapsulation of soluble bioactive substances for slow and targeted release.

Chapter 9: General discussion, conclusion and recommendation

9.1 GENERAL DISCUSSION

9.1.1 Production and characteristics of xylans from feedstocks found in South Africa

Water soluble xylan fractions were extracted from *E. grandis*, *P. patula*, bamboo and bagasse found in South Africa using mild alkali-low temperature methods. The solubility of these xylan fractions was modified by enzymatic methods to expand their functional properties, in particular, their use in precipitated form. The functionalisation of the extracted xylans in this manner could diversify the spectrum of the lignocellulosic products in the South African pulp and paper and timber industries by allowing production of high value products. However, the extraction, modification and utilisation of the xylans are processes that have not yet been developed for these South African feedstocks. As Gröndahl et al. (2003) explained the lack of utilisation of xylans in industrial applications is attributed to lack of well developed methods for extracting the xylans from lignocellulosic materials close to their native state. Hence, the study has created a basis for developing advanced methods for extraction and functionalisation of xylans for the South African feedstock, which will spare xylans from being disposed into process streams and bring about integrated lignocellulosic processing for production of multiple streams of high value products.

The extraction of the xylans was achieved by mild alkali-low temperature methods which were perceived to be selective for xylans compared to conventional wood pulping conditions (Chapter 3). In their native state, the xylans are water soluble and have an average degree of polymerisation of close to 200 sugar units (Sjöström, 1993) in hardwoods. However, as indicated by Timell (1967) and Wilkie (1979) the close association of the xylans with cellulose and lignin in the cell wall poses a challenge to isolate water soluble xylans for targeted modification. Consequently, extraction methods have to be carefully chosen to allow efficient xylan fractionation and addition of value. However, due to the differences in the structural and chemical composition of the feedstocks, the extraction efficiencies of the xylans from the feedstocks would be different. The results in Chapter 3 showed that the xylans in *P.*

patula were highly extractable compared to *E. grandis*, bagasse and bamboo (Chapter 3). However, the *P. patula* might not be an important source of xylans for industrial applications because of inherently low (7-10% dry weight) xylan content. Therefore, the mild alkali-low temperature methods need to be optimised for *E. grandis*, bagasse and bamboo feedstocks because of their high xylan content (Chapter 3).

The extent to which the xylans are extracted from the feedstocks has implications on the end use of the cellulolignin residues in integrated lignocellulose processing. The cellulolignin residues present new feedstock with new functionalities that would give options for production of speciality cellulosic, lignin and cellulose-lignin composites in the pulp and paper and timber industries. The high residual xylan content in the cellulolignin is important for subsequent use of the residue as a raw material for pulping. This is because the presence of the xylans in the pulp fibres is known to increase the pulp yield and enhances bonding strength of the pulp fibres during paper making (Ragauskas et al., 2006). The integration of the extraction of the xylans with production of speciality pulp or timber products would further open up multiple streams of high value lignocellulosic products from the South African feedstocks that could lead to establishment of biorefineries analogue to the petroleum refineries. The results presented in Chapter 3 suggest that more xylans remained in the cellulolignin residue of *E. grandis* and bagasse than in the cellulolignin residue of *P. patula* after the xylan extraction. Therefore, the over extraction of the xylan from the *P. patula* feedstock would compromise the possibility of co-generating the xylans with other cellulosic products.

9.1.2 Production of recombinant α -L-arabinofuranosidase in *Aspergillus niger*

A recombinant *Aspergillus niger* D15 strain system was developed through recombinant technology that selectively secreted α -L-arabinofuranosidase (AbfB) with polymeric xylan substrate specificity. The use of enzymes in lignocelluloses processing is not yet a common practice in South Africa for processing of lignocellulosic materials. The attributing factors include a lack of supply compounded by a lack of technical knowhow of using the enzymes. The α -L-arabinofuranosidases and α -D-glucuronidase with polymeric substrate specificity are important enzymes for

targeted modification of water soluble xylans (Chapters 4-8). Although the activity of the AbfB has been identified in a number of micro-organism (Biely, 2003), the extracellular production of the AbfB has been hampered by inefficient production systems and contamination with xylan main chain degrading enzymes and extracellular protease activities. The contributing factors have included inefficient secretion pathways and inefficient codon usage (Verdoes et al., 1995; Margolles-Clark et al., 1997; Punt et al., 2002). Therefore, to achieve selective production of the AbfB in *A. niger* (Chapter 4) is a breakthrough in advancing lignocelluloses processing. However, further development of the recombinant microbial systems is also needed to increase secretion quantities and over production of other side chain removing enzymes including α -D-glucuronidase (AguA) with polymeric xylan substrate specificity.

9.1.3 Role of side group removing enzymes in functionalisation of xylans

The use of *Eucalyptus grandis*, *Pinus patula*, *Bambusa balcooa* and bagasse feedstocks as raw materials for xylan extraction and of the AbfB and AguA has led into a new area of lignocelluloses processing.. In addition, the availability of AbfB and AguA presents opportunities to transform what initially was a wasted material during lignocelluloses processing such as kraft pulping, into a speciality coating material for production of novel lignocellulosic products (functional cellulosic and xylan products). Therefore, these side group removing enzymes have an emerging novel role in xylan processing, which arises from the increased demand for ‘green’ products that have less negative impact on the environment. In the study, the extracted xylans were substituted with arabinose and MeGlcA groups on the main xylan chain (Chapter 3), which provided opportunity for their targeted modification with the recombinant AbfB and purified AguA. Selective hydrolysis of the extracted xylans by the recombinant AbfB and the purified AguA modified xylan solubility and enhanced their functional properties as speciality coatings and encapsulation matrices (Chapter 5-8). The results showed that the AbfB and AguA provided flexible means of modifying xylan solubility properties that are tailor made for specific end uses (Chapters 7-8).

The recombinant AbfB and the purified AguA enhanced precipitation of water soluble xylans and their adsorption onto cellulosic materials. The xylans could be modified to

be used for production of cellulosic products with strong fibre to fibre bonding, new surface properties, increased water holding capacity, and reduced crystallinity by varying the side chain substitution pattern of the xylans (Linder et al., 2003^b, Kabel et al., 2007).

Through reduction of xylan solubility, the recombinant AbfB was capable of insolubilising arabinoglucuronoxylans from bamboo and bagasse into xylan nanohydrogel precipitates. Furthermore, the recombinant AbfB enhanced *in situ* formation of xylan nanohydrogels that successfully encapsulated and slowly released horse radish peroxidase (Chapter 8), whereas, both the recombinant AbfB and the purified AguA showed ability to enhance adsorption of the xylans onto cellulosic materials (Chapter 7 and 8). Such abilities offer opportunities of using the side chain removing enzymes to implant xylan hydrogel containing functional agents that would adsorb on cellulosic surfaces or any other surface for sustained release. This is a novel application of the enzymes and the xylans that will influence designing and manufacturing of lignocellulosic products and processes beyond the pulp and paper industry. For instance, there is potential application of the AbfB and AguA for modification and adsorption of xylans relevant to the coating industry, which could improve the wet end processes in manufacturing of pulp and paper. Furthermore, the interest in the use of the xylans is emerging in other industries other than the pulp and paper industries. For example, Österberg et al., (2001) showed that chemically modified xylans adsorbed on negatively charged mica surface, which suggest possibilities of using the modified xylans in concrete reinforcements and Silva et al. (2007) described the use of modified xylans for coating magnetite used in patients during MRI scanning process. In view of the fact xylans adsorbed on the surface of cellulosic materials introduces surface charges (Linder et al. 2003^{ab}); the modified xylans may also have a role in ionic exchange processes. Furthermore, the ability of the recombinant AbfB to alter the viscosity provides a new way of modifying xylans to be used as a thickening agent and emulsifier.

However, considering the substrate specificities of the recombinant AbfB and purified AguA, strategically, the recombinant AbfB would be a useful enzyme in production of xylan hydrogels for use as nano and microencapsulation matrices for slow release

or targeted delivery systems of bioactive agents because of the ability to precipitate xylans from a wide variety of sources. Precipitation of the xylans into nano and microencapsulation matrices has a role in particle engineering and nanotechnology. Moreover, the enzymatic method of transforming xylans into hydrogels would be most preferred over the chemical and physical methods because of reduced impact on the environment. On the other hand, the purified AguA released MeGlcA side chains from the xylans extracted *E. grandis*, *P. patula*, bagasse and bamboo feedstocks found in South Africa but did not cause any visible precipitation (Chapter 5 and 6) except of commercially obtained birch xylan (Chapter 5). However, the role of the purified AguA was prominent in enhancing *in situ* modification and adsorption of arabinoglucuronoxylans extracted from *P. patula*, bagasse and bamboo and glucuronoxylan extracted from *E. grandis* onto cotton lint (Chapter 7). Therefore, the purified AguA is an important enzyme in xylan coating processes where increased binding power of the xylans towards cellulosic materials or any other surfaces is critical compared to the recombinant AbfB.

The study showed the conditions for the enzymatic hydrolysis of the xylans could be altered to allow customised modification of the xylans including changing rheological and precipitation properties to different degrees (Chapter 5 and 6). The xylan concentration and hydrolysis time were found to be critical process parameters for controlled modification of the solubility properties of the xylans (Chapters 5 and 6), which also influenced enzyme aided adsorption of xylans onto cellulosic materials (Chapter 7) and formation of xylan nanohydrogels (Chapter 8). The functionalisation of the xylans by the AbfB and AguA was optimal under mild conditions, which is in contrast to the functionalisation of the xylans by physical or chemical methods, which often occurs at high temperatures ($\geq 100^{\circ}\text{C}$) and high alkaline conditions ($\text{pH} \geq 10$) (Westbye et al., 2006, Köhnke et al., 2008). Furthermore, the chemical and physical methods of functionalising the xylan are characterised with non-selectivity in modification of the xylan as opposed to the AbfB and AguA methods that provide opportunity for customised functionalisation of the xylan (Chapter 6 b). Therefore, from the technical, environmental and economic point of view, the use of the AbfB and AguA modification methods in xylan processing can be energy and chemical saving, which could also save the costs of disposing the chemical discharges while producing high value products.

Apart from providing new ways of processing lignocellulosic materials, the action of the AbfB and AguA would improve the performance of the existing lignocelluloses processes (Chapter 7). The removal of residual lignin is known to be hampered by crosslinkages formed between the xylan side chains and lignin upon its assembly onto pulp fibre surfaces. Therefore, the removal of the side chains from polymeric xylans provides the means to facilitate delignification of cellulosic materials and reduction of formation of hexenuronic acid in particular, from glucuronoxylans. The hexenuronic acids and residual lignin are both responsible for increased consumption of bleaching chemicals (Jeffries, 1994). The potential benefits to be realised from the enzymatic removal of the side chains from the polymeric xylans during kraft pulping process would include allowing customised adsorption of xylans onto cellulosic fibre surfaces with reduced effect of residual lignin and hexenuronic acids. Therefore, it is possible to integrate xylan extraction, enzyme modification and pulping to produce lignocellulosic products with high added value while reducing the impacts of the processing of lignocellulosic materials on the environment.

9.2 CONCLUSIONS

Xylans were extracted from *E. grandis*, *P. patula*, bamboo and bagasse found in South Africa using mild alkali-low temperature methods. The extracted xylans fractions were polymeric and were substituted with arabinose and MeGlcA side chains. The extraction of the xylans from *E. grandis*, *P. patula*, bamboo and bagasse found in South Africa will reduce degradation and wastage of the xylans during pulping and could improve the efficiency of the pulping process.

The recombinant α -L-arabinofuranosidase (AbfB) was selectively secreted extracellularly and free of detectable xylanase activity by the *A. niger* D15 [*abfB*] strain. The selective extracellular secretion of the recombinant AbfB was achieved by expressing the *abfB* gene in *A. niger* D15 strain under the transcriptional control of glyceraldehyde-3-phosphate dehydrogenase promoter (*gpd_P*) and glucoamylase terminator (*glaA_T*). The AbfB secretion was growth associated, which provided protein yield per unit biomass of 366.30 nkat g⁻¹ (DW) when recombinant *A. niger* was cultivated in 2xMM media enriched with 2% concentrated corn steep liquor. The AbfB was stable under cultivation, storage and application conditions at temperatures between 30-60°C and pH 3.0-6.0. The substrate specificity of the recombinant AbfB matched the structural and chemical composition of the polymeric xylans extracted from *P. patula*, bamboo and bagasse found in South Africa.

The selective removal of the arabinose from oat spelt xylan, bamboo and bagasse recombinant AbfB led to altered xylan viscosity and formation of insoluble xylan hydrogel precipitates. The selective removal of MeGlcA from birch xylan and the xylans extracted from the *E. grandis*, *P. patula*, bamboo and bagasse found in South Africa by the purified α -D-glucuronidase (AguA) from *S. commune*, was able to cause visible precipitation only of the birch xylan. The synergetic effect of the AbfB and AguA was more effective for the removal of arabinose side chains compared to the removal of the MeGlcA side chains.

The ability of the AbfB and AguA to reduce the solubility of the xylans was significantly influenced by xylan concentration, hydrolysis time and temperature. Customized modification of the xylan viscosity and precipitation of the xylan was

possible by performing the selective hydrolysis of the xylans by the recombinant AbfB and AguA at varied temperature between 30 and 50°C, enzyme dosage between 180 and 540 nkat g⁻¹_{substrate} and hydrolysis time between 1 and 16 h. The optimal conditions for maximum removal of 43% of available arabinose from oat spelt xylan by the recombinant AbfB were 14-16 h at 38-45°C with recombinant AbfB xylan specific dosage of 607.0 nkat substrate (g)⁻¹. Whereas, the optimal conditions for the purified AguA MeGlcA removal based on birch xylan, were 11 h at 38°C with AguA xylan specific dosage of 18 000 nkatg⁻¹_{substrate}. The optimal conditions for the efficient removal of the arabinose and MeGlcA by AbfB and AguA could be predicted by the quadratic models with regression coefficients of > 0.9.

The AbfB and AguA increased adsorption of water soluble xylans onto the cotton lint. The application of AguA was more effective in increasing adsorption of arabinoglucuronoxylans extracted from *P. patula*, bamboo and bagasse onto the cotton lint than the use of AbfB and a cocktail of the AbfB and AguA. The *in situ* modification and adsorption of xylans onto cellulosic material aided with the recombinant AbfB and AguA, introduced varying hydration and structural properties, which would suit specific industrial applications. Furthermore, the recombinant AbfB enhanced formation of xylan nanohydrogels during the selective hydrolysis of water soluble oat spelt xylan, which were spherical and exhibited negative zeta potential of up to -19 mV. The xylan nanohydrogels were successfully applied as encapsulation matrix for slow release of horse radish peroxidase (HRP) enzyme over a period of 180 min.

The selective hydrolysis of water soluble xylans by the recombinant AbfB enzyme and purified AguA allows customised modification of the functional properties of polymeric xylans from various South African feedstocks, which could diversify functional properties of xylans and advance processing of lignocelluloses. Therefore, the AbfB and AguA present a novel enzyme technology for production of precipitated xylan hydrogels from lignocellulosic materials for use as speciality additives, coating and implantation or encapsulation matrices as slow delivery systems with reduced impact on the environmental.

9.3 RECOMMENDATIONS

The success of the enzyme technology for modification of xylans would depend on the availability of xylans with structural and chemical composition that matches the substrate specificities of the recombinant AbfB and purified AguA. Although the Hoiye mild alkali–low temperature xylan extraction method enable the extraction of the xylans from *E. grandis*, *P. patula*, bamboo and bagasse feedstocks, it would be necessary to optimise the xylan extraction method to ensure that the xylan extraction chemicals, fractionation procedure and process conditions used for the extraction of the xylan that would suit the substrate specificity are efficient, cost effective and less polluting to the environment. Therefore, a life cycle assessments for extraction of the xylan from different feedstock in a biorefinery set up are needed.

The study has shown that the development of the enzyme technology has to be supported by availability of microbial enzyme production systems that would selectively overproduce the enzyme of interest. Further investigations are needed for the development of recombinant microbial system for over production of α -D-glucuronidase. In addition, the synergetic effect of the AbfB and AguA in selective hydrolysis of arabinoglucuronoxylans for optimal removal of both arabinose and MeGlcA side chain should be investigated further to identify appropriate mode of application for their optimal functioning.

The integration of xylan extraction and co-oproduction of other cellulosic products falls within the concept of biorefinery analogous to petroleum refinery, which would require reconfiguration and modification of the lignocelluloses processes and unit operations in the current lignocellulose processing set up. In particular the kraft pulp and paper production process need to be modified to accommodate pre-extraction, selective hydrolysis and re-introduction of the xylans in the production system. Therefore, it is important to determine optimal processing conditions for the pulp, including chemical and energy requirements and conditions for *in situ* enzyme modification and adsorption in the pulp process line. There is need for further development of the enzyme technology for aiding adsorption of xylans onto different cellulosic materials and non cellulosic materials. In addition, the conditions for

production of stable xylan nanohydrogels with respect to morphology and size should further be investigated to ensure wider application of the xylan nanohydrogels.

REFERENCES

- Akiyoshi, K., Kobayashi, S., Shichibe, S., Mix, D., Baudys, M., Kim, S.W., and Sunamoto, J. 1998. Self-assembled hydrogel nanoparticle of cholesterol-bearing pullulan as a carrier of protein drugs: Complexation and stabilization of insulin. *J. Control Release*. **54**:313-320.
- Al-Dajani, W.W., and Tschirner, U.W. 2008. Pre-extraction of hemicellulose and subsequent kraft pulping. Part I: alkaline extraction. *TAPPI J.* **7**(6):3-8.
- Alriksson, B., Rose, S.H., Van Zyl, W.H., Sjöde, A., Nilvebrandt, N., and Jönsson, L.J. 2009. Cellulase production from spent lignocellulose hydrolysates by recombinant *Aspergillus niger*. *Appl. Environ. Microbiol.* **75**: 2366-2374.
- Aoyama, M., and Seki, K. 1999. Acid catalysed steaming for solubilisation of bamboo grass xylan. *Biores. Technol.* **69**:91-94.
- Argyropoulos, D.S., and Menachem, S.B. 1997. Lignin. *Advan. Biochem. Engin. Biotechnol.* **57**:127-158.
- Arndt, T., and Zelm, R. 2008. New nanotechnology-produced compounds in papermaking applications: Review and first own experiences. *ZELLCHEMING Annul Meeting*. Fibres and Raw Material, Lecture Series. Das Papier Science and Technology pp 59-64. http://74.125.155.132/scholar?q=cache:kF-Xzg82FwgJ:scholar.google.com/&hl=en&as_sdt=2000. (Accessed 01 March, 2010).
- Atalla, R.H., and Isogai, A. 2005. Recent developments in spectroscopic and chemical characterisation of cellulose. In Dumitriu, S. (ed.) *Polysaccharides structural diversity and functional versatility*. Marcel Dekker. New York. pp 123-157.
- Bailey, M.J., Biely, P., and Pountanen, K. 1992. Interlaboratory testing of methods for assay of xylanase activity. *J. Biotechnol.* **23**:257-270.
- Bajpai, P. 2004. Biological bleaching. *Critic. Rev. Biotechnol.* **24**(1):1-58.
- Balakshin, M.Y., Capanema, E.A., and Chang, H.-M. 2008. Recent advances in the isolation and analysis of lignins and lignin-carbohydrate complexes. In Hu, T.Q. (ed). *Characterization of lignocellulosic materials*. Blackwell, Publishing
- Beldman, G., Searle-van Leeuwen, M.J.F., De Ruiter, G.A. Siliha, H.A., and Voragen, A.G.J. 1993. Degradation of arabinans by arabinases from *Aspergillus aculeatus* and *Aspergillus niger*. *Carbohydr. Polym.* **20**:159-168.
- Beldman, G., Schols, H.A., Pitson, S.M., Searle-van Leeuwen, M.J.F., and Voragen, A.G.J. 1997. Arabinans and arabinan degrading enzymes. *Advanc. Macromol. Carbohydr. Res.* **1**: 1-64.
- Benkő, Z., Andersson, A., Szengyel, Z., Gáspár, M., Réczey, K., and Ståhlbrand, H. 2007. Heat extraction of corn fibre hemicellulose. *Appl. Biochem. Biotechnol.* **136-140**:253-265.
- Berlanga-Reyes, C.M., Carvajal-Millán, E., Lizardi-Mendoza, Rascón-Chu, A., Marquez-Escalante, J.A., and Martínez-López, A.L. 2009. Maize arabinoxylan gels as protein delivery matrices. *Molecules* **14**:1475-1482.
- Beylot, M.-H., Emami, K., Mckie, V.A., Gilbert, H.J., and Pell, G. 2001. *Pseudomonas cellulose* expresses a single membrane bound glycoside hydrolase family 51 arabinofuranosidase. *Biochem. J.* **358**:599-605.

- Biely, P. 2003. Xylanolytic enzymes. In: Whitaker, R.J., Voragen, A.G.J. and Wong, D.W.S. (eds.) *Handbook of food enzymology*. pp 879-916. Marcel Dekker Inc, New York.
- Biely, P., Vrsanska, M., Kucar, S., 1992. Identification and mode of action of endo(1,4)- β - xylanases. In: Visser. J. Beldman, G., Xystersvan Someren, M.A. Voragen., A.G.J. (eds.). *Xylans and Xylanases*, Elsevier, Amsterdam. pp. 81-95.
- Biliaderis, C.G. 1995. Effect of arabinoxylans on bread-making quality of wheat flours. *Food Chem.* **53**:165-171.
- Bills, A.M., Falkehag, S.I., Kleppe, P.J., and Charleston, S.C. 1970. Methods for stabilising polysaccharides against alkaline attack. *United States Patent* No 3,532,596. United States Patent Office.
- Birgisson, H., Fridjonsson, O., Bahrani-Mougeot, F.K., Hreiddvidsson, G.O., Kristjansson, J.K., and Mattiasson, B. 2004. Thermostable α -L-arabinofuranosidase from a novel thermophilic bacterium. *Biotechnol Lett.* **26**:1347-1351.
- Blobleter, O. 2005. Hydrothermal degradation and fractionation of saccharides and polysaccharides. In Dimutriu, S. (ed). *Polysaccharides structural diversity and functional versatility*. 2nd edition. Marcel Dekker, New York. pp 893-936.
- Bradford, M.M. 1976. A rapid and sensitive method for quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**: 248-254.
- Brienzo, M., Siqueira, A.F., and Milagres, A.M.F. 2009. Search for optimum conditions of sugarcane bagasse hemicellulose extraction. *Biochem. Engin. J.* **46**:199-204.
- Browning, B.L. 1970. Wood Chemistry. In: Britt, K.W. (ed.). *Handbook of Pulp and Paper Technology*. Letion Educational Publishing Inc. pp3-12.
- Browning, B.L. 1967. Methods of wood chemistry. Vol II. Interscience Publishers. pp882.
- Brumer, H., Zhou, Q., Baumann, M.J., Carlsson, K., and Teeri, T.T. 2004. Activation of crystalline cellulose surfaces through the chemoenzymatic modification of xyloglucan. *J. Am. Chem. Soc.* **126**:5715-5721.
- Buchert, J., Sii-kaaho, M., Rättö, M., Viikari, L., and Bailey, M. 1993. Method and enzymatic preparation for treatment of cellulose pulp. *World Intellectual Property Organisation*. WO **93/11296**.
- Buchert, J., Teleman, A., Harjunpää, V., Tenkanen, M. Viikari, L., and Vuorinen, T. 1995. Effect of cooking and bleaching on the structure of xylan in conventional pine kraft pulp. *TAPPI J.* **78**: 125–130.
- Carlos, A., Chancón-Martínez, C.A., Anzola, J.M., Rojas, A., Hernández, F., Junca, H., Ocampo, W., and Portillo, P.D. 2004. Identification and characterization of the α -L-arabinofuranosidase B of *Fusarium oxysporum* f. sp. *Dianthi*. *Physiol. Molec. Plant Pathol.* **64**:201-208.
- Caruso, F., Trau, D., Möhwald, H., and Renneberg, R. 2000. Enzyme encapsulation in layer-by-layer engineered polymer multilayer capsules. *Langumuir* **16**:1485-1488.
- Carvajal-Millan, E., Gugliarelli, B., Belle, V., Rouau, X., and Micard, V. 2005. Storage stability of laccase induced arabinoxylan gels. *Polym.* **59**(2):181-188.
- Castanares, A., Hay, A.J., Gordon, A.H., MacCrae, S.I., and Wood, T.M. 1995. D-Xylan-degrading enzyme system from fungus *Phanerochaete chrysosporium*:

- Isolation and partial characterisation of an α -(4-*O*-methyl)-D-glucuronidase) *J. Biotechnol.* **43**:183-194.
- Cha, D., and Chinnan, M. 2004. Biopolymer-based antimicrobial packaging: A review. *Crit. Rev Food Sci Nutr.* **44** (4):223-237.
- Cipriani, T.R., Mellinger, C.G., De Souza, L.M., Baggio, C.H., and Freitas, S. 2008. Acidic heteroxylans from medicinal plants and their anti-ulcer activity. *Polym.* **74**:274-278.
- Clarke, J.H., Davidson, K., Rixon, J.E, Halstead, J.R., Fransen, M.P., Gilbert, H.J., and Hazlewood, G.P. 2000. Comparison of enzyme-aided bleaching of softwood paper pulp using combinations of xylanase, mannanase and α -galactosidase. *Appl. Microbiol. Biotechnol.* **53** (6):661-667.
- Coimbra, M.A., Waldron, K.W., and Selvendran, R.R. 1995. Isolation and characterisation of cell wall polymers from heavily lignified tissues of olive (*Olea europae*) seed hull. *Carbohydr. Polym.* **27**:285-294.
- Cole, S.M., Garb, J.E, and Woodson, L.P. 1992. Water insoluble polysaccharides hydrogel foam for medical applications. *US patent number* **5089 606**.
- Conesa, A., Punt, P.J., van Lwijk, N., and Van Den Hondel, C.A.M.J.J. 2001. The secretion pathway in filamentous fungi: A biotechnological view. *Fungal Genet. Biol.* **33**:155-171.
- Coviello, T., Matricardi, P., Marianecci, C., and Alhaique, F. 2007. Polysaccharides hydrogels for modified release formulations. *J. Control. Release.* **119**:5-24.
- Crous, J.M., Pretorius, I.S., and Van Zyl, W.H. 1996. Cloning and expression of the α -L-arabinofuranosidase gene (ABF2) of *Aspergillus niger* in *Saccharomyces cerevisiae*. *Appl. Microbiol. Biotechnol.* **46**: 256-260.
- Czernik, S., French, R., Feik, C., and Chornet, E. 1999. Production of hydrogen from hemicellulose-rich fractions generated through steam fractionation of biomass. http://www.anl.gov/PCS/acsfuel/preprint%20archive/Files/44_2_ANAHEIM_03-99_0240.pdf (Accessed 30th November, 2009).
- Dahlman, O., Jacobs, A., Sjöberg, J. 2003. Molecular properties of hemicelluloses located in the surface and inner layers of hardwood and softwood pulps. *Cellulose.* **10**:325-334.
- Dammström, S., Salmén, L., and Gatenholm, P. 2009. On the interactions between cellulose and xylan, a biomimetic simulation of the hardwood cell wall. *Biores.* **4** (1):3-14.
- Danielsson, S. 2007. Xylan reactions in Kraft cooking: process and product considerations. *Doctorial Thesis*. Royal Institute of Technology, Stockholm, Sweden. pp 84.
- Danielsson, S., Kisara, K., and Lindstrom, M.E. 2006. Kinetic study of hexenuronic and methylMeGlcA reactions in pulp and in dissolved xylan during kraft pulping of hardwood. *Industr. Engin. Chem. Res.* **45**(7): 2174-2178.
- Daus, S., and Heinze, T. 2009. Xylan-based nanoparticles: prodrugs for ibuprofen release. *Macromol. Biosci.* **10** (2):211-220.
- Debeche, T., Cummings, N., Connerton, I., Debeire, P., and Odonohoue, M.J. 2000. Genetic and biochemical characterisation of a highly thermostable α -L-arabinofuranosidase from *Thermobacillus xylanilyticus*. *Appl. Environ. Microbiol.* **66** (4):1734-1736.
- De Ioannes, P., Peirano, A., Steiner, J., and Eyzaguirre, J. 2000. An α -arabinofuranosidase from *Penicillium purpurogenum* production, purification, and properties. *J. Biotechnol.* **76**:253-258.

- De Lopez, S., Tissor, M., and Delmas, M. 1996. Integrated cereal straw valorization by an alkaline pre-extraction of hemicellulose prior to soda-anthraquinone pulping. Case study of barley straw. *Biomass and Bioenergy*. **10** (4):201-211.
- De Vries, R.P., Van de Vondervoot, P.J.I., Hendriks, L., Van De Belt, M., and Visser, J. 2002. Regulation of α -glucuronidase-encoding gene (*aguA*) from *Aspergillus niger*. *Molec. Gen. Genom.* **268**: 96-102.
- De Vries, R.P., and Visser, J. 2001. *Aspergillus* enzymes involved in degradation of plant cell wall polysaccharides. *Microbiol. Mol. Biol. Rev.* **65**:497-522.
- De Wet, B.J.M., Matthew, M.K.A., Storbeck, K.-H., and Van Zyl, W.H., and Prior, B.A. 2008. Characterization of a family 54 α -L-arabinofuranosidase from *Aureobasidium pullulans*. *Appl. Microbiol. Biotechnol.* **77**:975-983.
- De Wet, B.J.M., and Prior, B.A. 2004. Microbial α -glucuronidase. In Saha, B.C., and Hayashi, K. (eds). Lignocellulose Biodegradation. *ACS Sympos. Ser.* **889**:241-255.
- Dea, I.C.M., Rees, D.A., Beveridge, R.J., and Richard, G.N. 1973. Aggregation with change of conformation in solution of hemicellulose xylans. *Carbohydr. Res.* **29**:363-372.
- Dekker, R. F.H. 1989. Biodegradation of the hetero-1,4-linked xylans. In Lewis, N.G. and Paice, M.G. (eds). Plant cell wall. *ACS Symp. Ser.* **399**: 619-629.
- Department of Minerals and Energy (DME). 2004. Assessment of commercially exploitable biomass resources: bagasse, wood, and sawmill waste and pulp in South Africa. Capacity building in energy efficiency and renewable energy. *Report No-2.3.4-29*. pp 99.
- Domb, A.J., Kumar, N., Sheskin, T., Bentolila, A., Slager, J., and Teomim, D. 2002. Biodegradable polymers as drug carrier systems. In Dumitriu, S. (ed). *Polymeric biomaterials*. Marcel Dekker Inc. New York. pp 91-121.
- Dreschsler, U., Loth, F., and Hettrich., K. 2008. Stable hemicellulose paste, method for production thereof and use thereof. *USPTO Application#*: **20080199573**.
- Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A., and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. *Annal. Chem.* **28** (3):350-356.
- Dumitriu, S. 2002. Polysaccharides as biomaterials. In Dumitriu, S. (ed). *Polymeric biomaterials*. Marcel Dekker Inc. New York. pp 1-61.
- Dunn-Coleman N. S., Bodie E. A., Carter G. L. and Armstrong, G. L. 1992. Stability of recombinant strains under fermentation conditions. In: Kinghorn, J.R & Turner, G. (eds.) *Applied Molecular Genetics of Filamentous Fungi*. Blackie, Glasgow. pp 152-174.
- Duong, D.D. 1998. Adsorption analysis: equilibrium and kinetics. *Chem. Engin. Ser.* Vol. 2. Imperial College Press, London. pp 892.
- Du Toit, P.J., Olivier, S.P., and Van Biljon, P.L. 2010. Sugarcane bagasse as a possible source of fermentable carbohydrates. I. Characterization of bagasse with regard to monosaccharide, hemicellulose, and amino acid composition. *Biotechnol. Bioengin.* **26** (9):1071-1078.
- Ebringerová, A., and Heinze, T. 2000. Xylan and xylan derivatives- with valuable properties 1: Naturally occurring xylan structures, isolation procedures and properties. *Macromol. Rapid Comm* **21**:542-556.
- Ebringerová, A., Kardošová, A., Hromádková, Z, Malovíková, A., and Hříbalová, V. 2002. Immunomodulatory activity of acidic xylans in relation to their structural and molecular properties. *Int. J. Biol. Macrom.* **30**:1-6.
- Ebringerová, A. 2006. Structural diversity and application potential of hemicelluloses. *Macromol. Symp.* **232**: 1-12.

- Ebringerová, A., Hromádková, Z., Alfödi, J., and Hříbalová, V. 1998. The immunologically active xylan from ultrasound-treated corn cobs: extractability, structure and properties. *Carbohydr. Polym.* **37**: 231-239.
- Ebringerová, Hromádková, Z., and Heinze, T. 2005. Hemicellulose. *Adv. Polym. Sci.* **128**:1-68.
- Emmel, A., Mathias A.L., Wypych, F., and Ramos, L.P. 2003. Fractionation of *Eucalyptus grandis* chips by dilute acid-catalysed steam explosion, *Biores. Technol.* **86**:105–115.
- Eriksson, K.-E.L., Blanchette, R.A., and Ander, P. 1990. Microbial and enzymatic degradation of wood and wood components. In Timell, T.E. (ed). Springer Series in Wood Science. Springer-Verlag, Berlin, Germany. pp416.
- Fardim, P., and Durán, N. 2004. Retention of cellulose, xylan and lignin in kraft pulping of Eucalyptus studied by multivariate data analysis: influences on physicochemical and mechanical properties of pulp. *J. Braz. Chem. Soc.* **15**(4):514-522.
- Fengel, D., and Wegener, G. 1989. Wood chemistry, ultrastructure, reactions. Walter de Gruyter, Berlin. Germany. pp 613.
- Fengel, D., and Shao, X. 1985. Studies on the lignin of the bamboo species *Phyllostachys makinoi* Hay. *Wood Sci. Technol.* **19**(2):131-137.
- Fernández-Espinar, M.T., Peña, J.L., Piñaga, F., and Vallés, S. 1994. α -Arabinofuranosidase production by *Aspergillus nidulans*. *FEMS Microbiol. Lett.* **115**: 107-112.
- Ferraz, A., Baeza, J., Rodríguez, J., and Freer, J. 2000. Estimating the chemical composition of biodegradable Pine and Eucalyptus wood by DRIFT spectroscopy and multivariate analysis. *Biores. Technol.* **74**:201-212.
- Finnie, S.M., Bettege, A.D., and Morris, C.F. 2006. Influence of cultivar and environment on water-soluble and water insoluble arabinoxylans in soft wheat. *Cereal Chem.* **83**(6):617-623.
- Flipphi, M.J.A., Van Heuvel, M., Van Der Veen, P., Visser, J., and De Graaff, L.H. 1993. Cloning and characterisation of the *abfB* gene coding the major α -L-arabinofuranosidase (ABFB) of *Aspergillus niger*. *Curr. Genet.* **24**:525-532.
- Fras-Zemljić, L., Stenius, P., Laine, J., and Stata-Kleinschek, K. 2006. The effect of adsorbed carboxymethyl cellulose on the cotton fibre adsorption capacity for surfactant. *Cellulose*, **13**:655-663.
- Fritz, M., Ravanal, M.C., Braet, C.J., and Eyzaguirre, J. 2008. A family 51 α -L-arabinofuranosidase from *Penicillium purpogruenum*: purification, properties and amino acid sequence. *Mycol. Res.* **112**: 933-942.
- Fu, D., Mazza, G., and Tamaki, Y. 2010. Lignin extraction from straw by ionic liquids and enzymatic hydrolysis of the cellulosic residue. *J. Agric. Food. Chem.* **58**:2915-2922.
- Gabrielii, I., Gatenholm, P., Glasser, W.G., Jain, R.K., and Kenne, L., 2000. Separation, characterisation and hydrogel-formation of hemicellulose from aspen. *Carbohydr. Polym.* **43**:367-374.
- Garcia, R.B., Nagashima, J., T., Praxedes, A.K.C, Raffin, F.N., Moura, T.F.A.L., and Do Egito, E.S.T., 2001. Preparation of micro and nanoparticles from corn corbs xylan. *Polym. Bull.* **46**: 371-379.
- Garrote, G., Domínguez, H., and Parajo, J.C. 2004. Production of substituted oligosaccharides by hydrolytic processing of barley husks. *Ind. Eng. Chem. Res.* **43**:1608-1614.

- Gatenholm, P., Grondahl, M., Dammstrom, S., Eriksson, L. 2008. Polymeric film or coating comprising hemicelluloses. Patent Genius. <http://www.patentgenius.com/patent/7427643.html> (accessed 17/11/2009).
- Geng, Z.C., Sun, J.X., Liang, S F., Zhang, F.Y., Zhang, Y.Y., and Xu, F. 2006. Characterization of water-and alkali-soluble hemicellulosic polymers from sugarcane bagasse. *Int. J. Polym. Characterization* **11**:209-226.
- Gielis, J. 2002. Future possibilities for bamboo in European agriculture. *Oprins Plant Sint-Lenaartsesteenweg* 91 B-2310 Rijkvorschel. www.oprins.com/bamboonetwerk/publication/gielis/gielis01.pdf. (Accessed 17 November 2009).
- Gielkens, M., González-Candelas, L., Sánchez-Torres, P., Van De Vondervoort P., De Graaff, L., Visser, J., and Ramón, D. 1999. The *abfB* gene encoding the major α -L-arabinofuranosidase of *Aspergillus nidulans*: nucleotide sequence, regulation and construction of a disrupted strain. *Microbiol.* **145**:735-741.
- Gielkens, M.M.C., Visser, J., and de Graaff, L. 1997. Arabinoxylan degrading fungi: characterization of the arabinoxylan-arabinofuranohydrolase encoding genes from *Aspergillus niger* and *Aspergillus tubingensis*. *Curr. Genet.* **31**:22-29.
- Gierer, J. 1985. Chemistry of delignification. Part 1: General concept and reactions during pulping. *Wood Sci. Technol.* **19**:289-312.
- Gilead, S., and Shoham, Y. 1995. Purification and characterization of α -L-arabinofuranosidase from *Bacillus stearothermophilus* T-6. *Appl. Environ. Microbiol.* **61**:170-174.
- Glasser, W.G., Kaar, W.E., Jain, R.K., Sealy, J.E. 2000. Isolation options for non-specific heteropolysaccharides (HetPS). *Cellulose.* **7**:299-317.
- Gomori, G. 1955. Preparation of buffers for use in enzyme studies. In Colowick, S.P. and Kaplan, N.O. (eds.). *Method. Enzymol.* **1**:138-146.
- Gouka, R.J., Punt, P.J., and Van Den Hondel, C.A.M.J.J. 1997. Efficient production of secreted proteins by *Aspergillus niger*: progress, limitations & prospects. *Appl. Microbiol. Biotechnol.* **47**:1-11.
- Gouka, R.J., Punt, P.J., Hessing, G.M., Van Den Hondel, C.A.M.J.J. 1996. Analysis of heterologous protein production in defined recombinant *Aspergillus awamori* strains. *Appl. Environ. Microbiol.* **62**(6):1951-1957.
- Griebel, A., Lange, T., Weber, H., Milacher, W., and Sixta, H. 2006. Xylo-oligosaccharides (XOS) formation through hydrothermolysis of xylan derived from viscose process. *Macromol. Symp.* **232**:107-120.
- Gröndahl, M., and Gatenholm, P. 2005. Role of acetyl substitution in hardwood xylan. In Dumitriu, S. (ed) *Polysaccharides structural diversity and functional versatility*, Marcel Dekker, New York. pp509-514.
- Gröndahl, M., Eriksson, L., and Gatenholm, P. 2004. Material properties of plasticized hardwood xylans for potential application as oxygen barrier films. *Biomacromol.* **5**: 1528-1535.
- Gröndahl, M., Teleman, A., and Gatenholm, P. 2003. Effect of acetylation on the material properties of glucuronoxylan from aspen wood. *Carbohydr. Polym.* **53**: 359-366.
- Gurlal, P., Moodley, V., and Van Zyl, P.J. 2006. Evaluation of the possible impact of AspA salts and glucose concentration on mannanase and biomass production by *Aspergillus niger* (D15) in flasks. *CSIR /BIO/IR/PPD/2006/0023/B*, CSIR, pp 1-8.

- Gweyn, D.L. 1992. Foreign proteins. In: Kinghorn, J.R, and Turner, G. (eds.) *Applied Molecular Genetics of Filamentous Fungi*: Blackie, Glasgow. Pp 132-151.
- Gyenis, J., Feczko, T., Biro, E., Nemeth, A. S., Toth, J., Sisak, C. 2006. Functional nano/microparticles: properties, preparation and applications *Proceedings of Mini-Conference Biopowders, Properties, Production & Handling*. SIK, Gothenburg, Sweden. 29-30 June, 2006. pp 8-15.
- Habibi, Y., and Vignon, M.R. 2005. Isolation and characterization of xylans from seed pericarp of *Argania spinosa* fruit. *Carbohydr. Res.* **340**:1431-1436.
- Haimer, E., Wendland, M., Potthast, A., Rosenau, T., and Liebner, F. 2008. Precipitation of hemicelluloses from DMSO/water mixtures using carbon dioxide as an antisolvent. *J. nanomaterials*. DOI: 10.1155/2008/826974. 2008: 1-5.
- Håkansson, H., Germgård, U., and Sens, D. 2005. Influence of xylan on the degradability of laboratory kraft pulps from hardwood and reed canary grass in acid hydrolysis. *Cellulose*. **12**:621-628.
- Haltrich, D., Nidetzky, B., Kulbe, K.D., Steiner, W., and Župančič, S. 1996. Production of fungal xylanases. *Bioresour. Technol.* **58**:137-161.
- Hammes, B., Ruiz, R. Scarlata, C., Sluiter, A., Sluiter, J., and Templeton, D. 2005. Preparation of samples for compositional analysis. Laboratory Analytical Procedure (LAP), version 2005. NREL Biomass Program. National Bioenergy Center.
- Hans, N. L., and Lowman, A.M. 2002. Biodegradable nanoparticles for drug delivery and targeting. *Curre. Opin. Solid State Material. Sci.* **6**:319-327.
- Hansson, J. 1970. Sorption of hemicelluloses on cellulose fibres. Part 3. The temperature dependence on sorption of birch xylan and pine glucomannan at kraft pulping conditions. *Svensk Papperstidning*, **73**(3):49-53.
- Harvey, L.M., and McNeil, B. 1994. Liquid fermentation systems and product recovery of *Aspergillus*. In: Smith J.E. (ed.) *Aspergillus-Biotechnology*. pp 219-261.
- Hatti-Kaul, R., and Mattiasson, B. 2001. Downstream processing in biotechnology. In: Ratledge, C. and Kristansen, B. (eds.) *Basic Biotechnology*. Cambridge, London. pp187-211.
- Haun, J.L. 1970. Hemicellulose. In Britt K.W. (ed.) *Handbook of Pulp and Paper Technology*. Litton Educational Publishing Inc. pp 25-31.
- Hazlewood, G.P., and Gilbert, H.J. 1993. Molecular biology of hemicellulases. In Coughlan, M.P., and Hazlewood, G.P (eds). *Hemicellulose and Hemicellulase*. Portland Press, London. pp 103-126.
- Henriksson, Å., and Gatenholm, P. 2002b. Surface properties of CTMP fibers modified with xylans. *Cellulose*. **9**:55-64.
- Henriksson, Å. and Gatenholm, P. 2001. Controlled assembly of glucuronoxylans onto cellulose fibres. *Holzforsch.* **55**(5): 494-502
- Henriksson, Å. and Gatenholm, P. 2002a. Adsorption on the properties of cellulose fibres *Cellul.*, **15** (4):537-546.
- Hettrich, K., Fischer, S., Schröder, N., Engelhardt, J. 2006. Derivatization and characterization of xylan from oatspelt. *Macromol. Symp.* **232**:37-48.
- Hille, A., Neu, T.R., Hempel, D.C. and Horn, H. 2005. Oxygen profiles and biomass distribution in biopellets of *Aspergillus niger*. *Biotechnol. Bioeng.* **92** (5):614-623.

- Höije, A., Gröndahl, M., Tømmeraas, K. and Gatenholm, P. 2005. Isolation and characterization of physicochemical and material properties of arabinoxylans from barley husks. *Carbohydr. Polym.* **61**: 266–275.
- Höije, A., Sternemaim, E., Heikkinen, S. Tenkanen, M., and Gatenholm, P. 2008. Material properties of films from enzymatically tailored arabinoxylans. *Biomacromol.* **9**:2042-2047.
- Hornig, S., and Heinze, T. 2008. Efficient approach to design stable water dispersible nano particles of hydrophobic cellulose esters. *Biomacromol.* **9**:1487-1492.
- Hornig, S., Bunjes, K., Heinze, T. 2009. Preparation and characterization of nano particles based on dextran-drug conjugates. *J. Colloid interface Sci.* **338**:56-62.
- Horvath, A.L. 2006. Solubility of structurally complicated materials. I. wood. *J. Phys. Chem. Data.* **35** (1):77-92.
- Howard, R.L., Abotsi, E., Van Rensburg, E.L.J., and Howard, S. 2003. Lignocellulosic biotechnology: issues of bioconversion and enzyme production. *Afr. J. Biotechnol.* **12** (2):602-619.
- Hult, E.-L., Larsson, P.T., and Iversen, T. 2000. A comparative CP/MAS ¹³C-NMR study of cellulose structure in spruce wood and kraft pulp. *Cellulose* **7**:35-55.
- Inman-Bamber, N.G., Muchow, R.C., Robertson, M.J. 2002. Dry matter partitioning of sugarcane in Australia and South Africa. *Field Crop Res.* **76**:71-84.
- Izydorczyk, M.S., and Billaderis, C.G. 1995. Cereal arabinoxylans: advances in structure and physicochemical properties. *Carbohydr. Polym.* **28**:31-48.
- Izydorczyk, M.S., Billaderis, C.G., and Bushuk, W. 1991. Physical properties of water-soluble pentosans from different wheat varieties. *Cereal Chem.* **68** (2): 145-150.
- Jacobs, A., Larsson, P.T., and Dahlman, O. 2001. Distribution of uronic acid in xylans from various species of softwood and hardwood as determined by MALDI mass spectrometry. *Biomacromol.* **2** (3):979-990.
- Jain, R.K., Sjöstedt, M., and Glasser, W.G. 2001. Thermoplastic xylan derivatives with propylene oxide. *Cellulose.* **7**:319-336.
- James, E.R. 2006. Optimizing HBsAg by recombinant *Aspergillus niger*. MSc. Thesis. Department of Process Engineering. Stellenbosch University. RSA.
- Jarai, G., and Buxton, F. 1994. Nitrogen, carbon, and pH regulation of extracellular acidic proteases of *Aspergillus niger*. *Curr. Genet.* **26** (3):238-244.
- Jeffries, T.W., 1994. Biodegradation of lignin and hemicelluloses. In Ratledge, C. (ed). *Biochemistry of Microbial Degradation*. Kluwer Academic Publisher, The Netherlands. pp 233-277.
- Jiang, Z.H., Bouchard, J., and Berry, R. 2006. Evidence of the formation of lignin-hexenuronic acid-xylan complexes during modified craft pulping process. *Holzforsch.* **60**:137-142.
- Jönsson, A.-S., Nordin, A.-K., and Wallberg, O. 2008. Concentration and purification of lignin in hardwood kraft pulping liquor by ultrafiltration and nanofiltration. *Chem. Engin. Res. Design.* **86** (11):1271-1280.
- Joseleau, J.P., Comtat, J., and Ruel, K. 1992. Chemical structure of xylans and their interaction in the plant cell walls. In Visser, J., Beldman, G., Kusters-van Someren, M.A., and Voragen, A.G.J. (eds) *Xylans and xylanases*. Elsevier Science Publisher. pp 1-15.
- Kabel, M.A., Van Den Borne, H., Vincken, J.-P., Voragen, A.G.J., and Schols, H.A. 2007. Structural differences of xylan affect their interaction with cellulose. *Carbohydr. Polym.* **69**:94-105.

- Kaji, A., 1984. L-arabinosidases. *Adv. Carbohydr. Chem. Biochem.* **47**: 383-394.
- Kajiwara, K., and Miyamoto, T. 2005. Progress in structural characterization of functional polysaccharides. In Dumitriu, S. *Polysaccharides: Structural diversity and functional versatility*. Marcel Dekker, New York. pp 1-40.
- Kolenová, K., Ryabova, O., Vršanská, M., and Biely, P. 2010. Inverting character of family GH115 α -glucuronidases. *FEBS Letters*. Article in press.
[Doi:10.1016/j.febslet.2010.08.031](https://doi.org/10.1016/j.febslet.2010.08.031).
- Kaneko, S., Arimoto, M., Ohba, M., Kobayashi, H., Ishii, T., and Kusakabe, I. 1998. Purification and substrate specificities of two α -L-arabinofuranosidases from *Aspergillus awamori* IFO 4033. *Appl. Environ. Microbiol.* **64**:4021–4027.
- Kayserilioglu, B.S., Bakir, U., Yilmaz, L., Akkas, N. **2003**. Use of xylan, an agricultural by-product, in wheat gluten based biodegradable films: mechanical, solubility and water vapor transfer rate properties. *Biores. Technol.* **87**(3):239-246.
- Kennedy, M., and Krouse, D. Strategies for improving fermentation medium performance: a review. *J. Industr. Microbiol. Biotechnol.* **23**(6):456-475.
- Khandke, K.M., Vithayathil, P.J., and Murthy, S.K. 1989. Purification and characterisation of an α -D-glucuronidase from thermophilic fungus *Thermoascus aurantiacus*. *Arch. Biochem. Biophys.* **274** (2): 511-517.
- Kim, K.H., Tucker, M.P., Keller, F.A., Aden, A., and Nguyen, Q.A. 2001. Continuous countercurrent extraction from pretreated wood residues. *Appl. Biochem. Biotechnol.* **91-93**:253-267.
- Kinghorn, J.R., and Unkles, S.E. 1994. Molecular Genetics and Expression of Foreign Proteins in the Genus *Aspergillus*. *Biotechnology Handbooks*. Plenum publishing corporation. 7: pp65.
- Kirk, T.K., and Jeffries, T.W. 1996. Roles of microbial enzymes in Pulp and paper. In Jeffries, T.W., and Viikari, L. (eds). *Enzymes for pulp and paper processing*. *ACS Symp. Ser.* **655**:2-14.
- Kishida, A., and Ikada, Y. 2002. Hydrogels for biomedical and pharmaceutical applications. In Dumitriu, S. (ed). *Polymeric biomaterials*. Marcel Dekker Inc. New York. pp 133-147
- Kitchaiya, P., Intanakul, P., and Krairish, M. 2003. Enhancement of enzymatic hydrolysis of lignocellulosic waste by microwave pre-treatment under atmospheric pressure. *J. Wood Chem. Technol.* **23**(2):217-225.
- Kleinert, T.N. 1966, Mechanism of alkaline delignification II: Free radical reactions. *TAPPI* **49** (3):126-130
- Köhnke, T., and Gatenholm, P. 2007. The effect of controlled glucuronoxylan adsorption on drying-induced strength loss of bleached softwood pulp. *Nordic Pulp Paper Res. J.* **22** (4):508-515.
- Köhnke, T., Brelid, H., and Westman, G. 2009. Adsorption of cationised barley husk xylan on kraft pulp fibres: influence of degree of cationisation on adsorption characteristics. *Cellulose*. **16**:1109-1121.
- Köhnke, T., Pujolras, C., Roubroeks, J.P., and Gatenholm, P. 2008. The effect of barley husks arabinoxylan adsorption on the properties of cellulose fibres *Cellulose*. **15** (4):537-546.
- Kormelink F.J.M., and Voragen, A.G.J. 1993. Degradation of different [(glucurono)arabino]xylans by a combination of purified xylan-degrading enzymes. *Appl. Microbiol. Biotechnol.* **38**(5): 688-695

- Kormelink, F.J., Gruppen, H., and Voragen, A.G. 1993. Mode of action of (1→4)-β-D-arabinoxylan arabinofuranohydrolase (AXH) and α-L-arabinofuranosidases on alkali-extractable wheat-flour arabinoxylan. *Carbohydr. Res.* **249**:345–353.
- Koseki T., Miwa, Y., Mese, Y., Minayaga, A., Fushinobu, S., Wakagi, T., Shoun, H., Matsuzawa, H., and Hashizume, K. 2006. Mutational analysis of *N*-glycosylation recognition sites on the biochemical properties of *Aspergillus kawachii*, α-L-arabinofuranosidase 54. *Biochem. Biophys. Acta* **1760**: 1458-1464.
- Koseki, T., Okuda, M., Sudoh, S., Kizaki, Y., Iwano, K., Aramaki, I., and Matsuzaya, H. 2003. Role of two α-L-arabinofuranosidase in arabinoxylan degradation and characterization of the encoding genes from shochu koji molds, *Aspergillus awamori*. *J. Biosci. Bioeng.* **96** (3):232-241.
- Kruger, N.J. 1996. The Bradford method for protein quantification. In Walker J.M. (Ed.). The protein protocols handbook, 2nd Edition, Humana Press Inc. Totowa, N.J. pp15-20
- Kuhad, R.C., Singh, A., Eriksson, K.-E.L., 1997. Microorganisms and enzymes involved in the degradations of plant fibres cell walls. In: Eriksson, K.E.L. (ed) Biotechnology in the Pulp and Paper Industry-*Advanc. Biochem. Engin. Biotechnol.* **57**:45-125.
- Kumar, M.N.V.R. 2000. Nano and microparticles as controlled drug delivery devices. *Pharm. Pharmaceut. Sci.* **3** (2):234-258.
- Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**:680-685.
- Laganón, J.M., Cabedo, L. Cava, D., Feijou, J.L., Gavara, R., and Gimenez, E. 2005. Improving package food quality and safety. Part 2. *Nanocomposite Food Additives and Contaminants* **22** (10):994-998.
- Lahaye, M., Rondeau-Mouro, C., Deniaud, E., and Buleon, A. 2003. Solid-state ¹³C NMR spectroscopy studies of xylans in the cell wall of *Palmaria palmata* (L. Kuntze, Rhodophyta). *Carbohydr. Res.* **338**:1559-1569.
- Larsson, P.T., Hult, E-L., Wickholm, K., Pettersson, E., and Iversen, T. 1999. CP/MAS ¹³C-NMR spectroscopy applied to structure and interaction studies on cellulose I. *Solid State Nuclear Magnetic Resonance* **15**:31-40.
- Lawoko, M., Henriksson, G., and Gellerstedt, G. 2006b. Characterization of lignin carbohydrate complexes from spruce sulphite pulp. *Holzforch.* **60**:162-165.
- Lawoko, M., Henriksson, G., and Gellerstedt, G. 2006a. Characterisation of lignin carbohydrate complexes (LCCs) of spruce wood (*Picea abies* L.) isolated with two methods. *Holzforch.* **60**:156-161.
- Lee, A.H., Doherty, T.V., Linhardt, R.J., and Dordick, J.S. 2009. Ionic liquid-mediated selective extraction of lignin from wood leading to enhanced enzymatic cellulosic hydrolysis. *Biotechnol Bioeng.* **102** (5):1358-1376.
- Lee, R.C., Burton, R.A., Hrmova, M., and Fincher, G.B. 2001. Barley arabinoxylan arabinofuranohydrolases: purification, characterization and determination of primary structures from cDNA clones. *Biochem. J.* **356**:181-189.
- Lee, S.B., Bogaard, J., and Feller, R.L. 1989. Darkening of paper following exposure to visible and near-ultra violet radiation. *J. Americ. Instit. Conserv.* **28** (1):1-18.
- Levasseur, A., Benoit, I., Asther, M., and Record, E. 2004. Homologous expression of feruloyl esterase B from *Aspergillus niger* and characterization of the recombinant enzyme. *Protein Expres. Purif.* **37** (1):126-133.

- Li, J., and Gellerstedt, G. 1997. The contribution to Kappa number from hexenuronic acid groups in pulp xylan. *Carbohydr. Res.* **302**:213-218.
- Li, J., Kisara, K., Danielsson, S., Lindström, M.E., and Gellerstedt, G. 2007. An improved methodology for quantification of uronic acid units in xylans and other polysaccharides. *Carbohydr. Res.* **342** (11):1442-1449.
- Liitia, T., Maunu, S.L., and Hortling, B. 2001. Solid state NMR on inhomogeneous structure of fibre wall in kraft pulp. *Holzforsch.* **55**:503-510.
- Lindblad, M.S., Ranucci, E., and Albertsson, A.-C. 2001. Biodegradable polymers from renewable sources. New hemicellulose-based hydrogels. *Macromol. Rapid Commun.* **22**:962-967.
- Lindblad, M.S. 2003. Strategies for building polymers from renewable sources: using prepolymers from steam treatment of wood and monomers from fermentation of agricultural products. Doctoral Thesis, KTH Fibre and Polymer Technology Royal Institute of Technology, Stockholm.
- Lindblad, M.S., and Albertsson, A.-C., 2005. Chemical modification of hemicellulose and gums. In Dumitriu, S. (ed) *polysaccharides structural diversity and functional versatility*. Marcel Dekker, New York. pp 491-508.
- Linder, Å., Bergamon, R., Bodin, A., and Gatenholm, P. 2003a. Mechanism of assembly of xylan onto cellulose surfaces. *Langmuir*. **19**:5072-5077.
- Linder, Å., Roubrocks, J.P. and Gatenholm, P. 2003b. Effect of ozonation on assembly of xylans. *Holzforsch.* **57**:496-502.
- Lisboa, S.A., Evtuguin, D.V., Neto, C.P., and Goodfellow, B.J. 2005. Isolation and structural characterisation of polysaccharides in *Eucalyptus globulus* kraft black liquors. *Carbohydr. Polym.* **60**:77-85.
- Liu, C., and Wyman, C.E. 2004. The effect of flow rate of very dilute sulphuric acid on xylan, lignin and total mass from corn stover. *Ind. Eng. Chem. Res.* **43**: 2871-2788.
- Lundqvist, J., Teleman, A., Junel, L., Zacchi, G., Dahlman, O., Tjerneld, F., and Stålbrand, H. 2002. Isolation and characterisation of galactoglucomannan from Spruce (*Picea abies*). *Carbohydr. Polym.* **48**:29-39.
- Luonteri, E., Siika-aho, M., Tenkanen, M., and Viikari, L. 1995. Purification and characterization of three arabinofuranosidases from *Aspergillus terreus*. *J. Biotechnol.* **38**:279-291.
- Luonteri, E., Beldman, G., and Tenkanen, M. 1998. Substrate specificities of *Aspergillus terreus* α -arabinofuranosidases. *Carbohydr. Polym.* **37**: 131-141.
- Malvern Instruments Ltd. 2004. What is the Zetasizer Nano? Zetasizer Nano Series user manual, *MAN03171* (1): 2-1-2-3.
- Manin, C., Shareek, F., Morosoli, R., and Kluepfel, D. 1994. Purification and characterization of an alpha-L-arabinofuranosidase from *Streptomyces lividans* 66 and DNA sequence of the gene (abfA). *Biochem. J.* **302**(2): 443-449.
- Margolles, A., and De Los Reyes-Gavilán, C.G. 2003. Purification and functional characterization of a novel α -L- arabinofuranosidase from *Bifidobacterium longum* B667. *Appl. Environ. Microbiol.* **69** (9):5096-5103.
- Margolles-Clark, E., Ilmén, M., and Penttilä, M. 1997. Expression patterns of ten hemicellulase genes of the filamentous fungus *Trichoderma reesei* on various carbon sources. *J. Biotechnol.* **57**:167-179.
- Margolles-Clark, E., Tenkanen, M., Nakari-Setälä, T., and Penttilä, M. 1996. Cloning of genes encoding α -L-arabinofuranosidase and β -xylosidase from *Trichoderma reesei* by expression in *Saccharomyces cerevisiae*. *Appl. Environ. Microbiol.* **62**(10): 3840-3846.

- Martin, C., Galbe, M., Nilvebrant, N.-O., Jönsson, L.J. 1998. Comparisson of fermentability of enzymatic hydrolyzate of sugarcane bagasse pretreated by steam explosion using different impregnating agents. *Appl. Biochem. Biotechnol.* **98-100**:699-716.
- Maunu, S.L. 2002. NMR studies of wood and wood products. *Progr. Nucl. Magnet. Resp. Spectr.* **40**:151-174.
- Maunu, S.L. 2008. ¹³C CPMAS NMR Studies of wood, cellulose fibers, and derivatives. In Hu, T.Q. (ed). *Characterization of lignocellulosic material*. Blackwell Publishing Ltd. Iowa, USA. pp 227-248.
- Maurer, H.W., and Kearney, R.L. 1998. Opportunities and challenges for starch in the paper industry *Starch/ Stärke* **50** (9):396-402.
- McIlvaine, T.C. 1921. A buffer solution for colorimetric comparison. *J. Biol. Chem.* **49**:183-186.
- McNeil, M., Darvill, A.G., Fry, S.C., and Albersheim, P. 1984. Structure and function of the primary cell wall of plants. *Ann Rev. Biochem.* **33**: 625-663.
- Me´ndez-Alcaraz, J.M., Johner, A., and Joanny, J. F. 1998. Density profiles and interaction between irreversibly adsorbed polymer layers. *Macromol.* **31**:8297-8304.
- Meller, A., 1964. Retention of polysaccharides in kraft pulping. III. Sheet and fiber strength properties and water retention capacity of kraft and borohydride kraft pulps. *TAPPI.* **47** (10): 634-639.
- Meller., A. 1965. The retake of xylan during alkaline pulping. *Holzforsch.* **19**:118-24.
- Metz, B., and Kossen, N.W.F. 1977. The growth of molds in the form of pellets-a literature review. *Biotechnol. Bioengin.* **19** (6):781-799.
- Miller, G.L. 1959. Use of dinitrosalicylic reagent for determination of reducing sugars. *Anal. Chem.* **31**: 426-428.
- Miyanaga, A., Koseki T., Matsuzawa, H., Wakagi, T, Shoun H., and Fushinobu S. 2004. Crystal structure of family 54- α -L-arabinofuranosidase reveals a novel carbohydrate –binding module that can bind arabinose. *J. Biol. Chem.* **279** (43): 44907-44914
- Miyanaga, A., Koseki, T., Miwa, Y., Mese, Y. Nakamura, S., Kuno, A., Hirabayashi, J., Matsuzawa, H., Wakagi, T., Shoun, H., and Fushinobu, S. 2006. The family 42 carbohydrate –binding module of family 54 α -L-arabinofuranosidase specifically binds arabinofuranose side chain of hemicellulose. *Biochem. J.* **399**:503-511
- Montgomery, D.C. 2005. Design and analysis of experiments. John Wiley&Sons, Inc. pp280.
- Morrison, I.M. 1974. Structural investigations on the lignin-carbohydrate complexes. *Biochem. J.* **139**:197-204.
- Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y.Y., Holtzapple, M., and Ladisch, M., 2005, Features of promising technologies for pretreatment of lignocellulosic biomass. *Bioresour. Technol.* **96**:673-686.
- Muller, R.H., Jacobs, C., Kayser, O, 2001. Nanosuspension as particulate drug formulations in therapy-rationale for development and what we can expect for the future. *Advanc. Drug Deliv.* **47**:3-19.
- Nagashima, T., Oliveira, E.E., Da Silva, A.E., Marcelini, H.R., Gomes, M.C.S., Aguiar, L.M., De Arúajo, I.B., Soares, L.A.L., De Oliveira, A.G., and Do Egito, E.S.T. 2008. Infuence of the lipophilic external phase composition on the preparation and characterisation of xylan microcapsules-Technical note. *AAPS PharmSciTech* **9** (3):814-817.

- Nagy, T., Nurrizzo, D., Davies, G.J., Biely, P., Lakey, J.H., Bolam, D.N., and Gilbert, H.J. 2003. The α -glucuronidase, Glc A67A of *Cellvibrio japonicas* utilizes the carboxylate and methyl groups of aldobiouronic acid as important substrate recognition determinant. *J. Biol. Chem.* **278** (22):20286-20292.
- Nagy, T., Emami, K., Fontes, C.M.G.A., Ferreira, L.M.A., Humphry, D.R., and Gilbert, H.J. 2002. The membrane -bound α -glucuronidase from *Pseudomonas cellulosa* hydrolyzes 4-O-methyl-D-glucuronoxyllogosaccharides but not 4-O-methyl-D-glucuronoxylan. *J. Bacteriol.* **184** (17):4925-4929.
- Nakamura, A., Miyafuji, H., Saka, S., Mori, M., and Takahashi, H. 2010. Recovery of cellulose and xylan liquefied in ionic liquids by precipitation in anti-solvents *Holzforsch.* **64**:77-79.
- Nelson, K.L. 2007. Enhanced performance and functionality of Titanium dioxide paper making pigments with controlled morphology and surface coating. *PhD Dissertation*. Georgia institute of Technology. pp208.
- Nogawa, M., Kenji, Y., Tomioka, A., Okada, H., and Morikawa, Y. 1999. An α -L-arabinofuranosidase from *Trichoderma reesei* containing a non-catalytic xylan binding domain. *Appl. Environ. Microbiol.* **65** (9):3964-3968.
- Numan, M.T., and Bhosle, N.B. 2006. α -Arabinofuranosidases: the potential applications in biotechnology. *J. Ind. Microbiol. Biotechnol.* **33**:247-260.
- O'Donnell, D., Wang, L., Xu, J., Ridgway, D., Gu T., and Moo-Young, M. 2001. Enhanced heterologous protein production in *Aspergillus niger* through pH control of extracellular protease activity. *Biochem. Eng. J.* **8**:187-193.
- Oliveira, L., Evtuguin, D.V., Cordeiro, N., Silvestre, A.J.D., and Da Silva, A.M.S. 2008. Chemical composition and lignin structural features of banana plant leaf sheath and rachis. In Hu, T.Q. (ed). *Characterisation of lignocellulosic materials*. Wiley-Blackwell: pp 171-188.
- Österberg, M., Láine, J., Stenius, P., Kumpulainen, A., and Claesson, P.M. 2001. Forces between xylan-coated surfaces charge density: Effect of polymer charge and background electrolyte. *J. Colloid. Intref. Sci.* **242** (1):56-69.
- Palm, M., and Zacchi, G. 2003. Extraction of hemicellulosic oligosaccharides from spruce using microwave oven or steam treatment. *Biomacromol.* **4**:617-623.
- Palm, M., and Zacchi, G. 2004. Separation of hemicellulosic oligomers from steam treated spruce wood using gel filtration. *Separat. Purif. Technol.* **36**:191-201.
- Pan, X., Arato, C., Gilkes, N., Gregg, D., Mabey, W., Pye, K., Xiao, X., Zhang, X., and Saddler, J., 2005. Biorefining of softwoods using ethanol organosolv pulping: Preliminary evaluation of process streams for manufacture of fuel-grade ethanol and co-products, *Biotech. Bioeng.* **90** (4):473-481.
- Park, S., and Kazlauskas, R.J. 2003. Biocatalysts in ionic liquids-advantages beyond green technology. *Curr. Opin. Biotechnol.* **14** (4):432-437.
- Patel, T.R., Harding, S.E., Ebringerova, A., Deszzyński, M., Hromadkova, Z., Togola, A., Paulsen, B., S., Morris, G.A., and Rowe, A.J. 2007. Weak self-association in carbohydrate system. *Biophysical J.* **93**:741-749.
- Paterson-Jones, J.C. 1989. Biological utilization of bagasse, a lignocellulose waste In Paterson-Jones, J.C. (ed). *National Scientific Programmes Unit: CSIR, SANSP Report 149*. Foundation Research Development. CSIR. pp 119.
- Pazouki, M., and Panda, T. 2000. Understanding the morphology of fungi. *Bioproc. Biosys. Eng.* **22** (2.):127-143.

- Pedersen, H., Beyer, M., and Nielsen, J. 2000. Glucoamylase production in batch, chemostat and fed batch cultivations by industrial strain of *Aspergillus niger*. *Appl. Microbiol. Biotechnol.* **53**:272-277.
- Peng, F., Ren, J.-L., Xu, F., Bian, J., Peng, P., and Sun R. C. 2009. Comparative study of hemicelluloses obtained by graded ethanol precipitation from sugarcane bagasse. *J. Agric. Food Chem.* **57**:6305-6317.
- Peppas, N.A., Bures, P., Leobandung, W., and Ichikawa, H. 2000. Hydrogels in pharmaceutical formulations. *Eur. J. Pharm. Biopharm.* **50**: 27-46.
- Peppas, L.B. 1997. Polymers in controlled drug delivery. *Med. Plastics Biomater. Magaz.* pp 34-46.
- Persson, T., Jönsson, A.-S., and Zacchi, G. 2005. Fractionation of hemicelluloses by membrane filtration. *14th European Biomass Conference*, 17-21 October, 2005, Paris, France.
- Pettersen, R.C. 1984. The chemical composition of solid wood. In: Rowell, R.M. (ed). *The chemistry of solid wood. Advanc. in Chem. Series* **207**:57-126.
- Pinto, P.C., Evtuguin, D.V., and Neto, C.P. 2005. Structure of hardwood glucuronoxylans: modifications and impact on pulp retention during wood kraft pulping. *Carbohydr. Polym.* **60**:489-497.
- Plüddemann, A., and Van Zyl, W.H. 2003. Evaluation of *Aspergillus niger* as host for virus-like particle production, using the hepatitis B surface antigen as a model. *Curr. Genet.* **43**: 439-446.
- Polizeli, M.L.T.M., Rizzatti, A.C.S., Monti, R., Terenzi, H.F., Jorge, J.A., and Amorim, D.S. 2005. Xylanases from Fungi: Properties and industrial applications. *Appl. Microbiol. Biotechnol.* Min-Review **67**:577-591.
- Pu, Y. Jiang, N., and Ragauskas, A.J. 2007. Ionic liquids as a green solvent for lignin. *J. Wood Chem. Technol.* **27**: 23–33.
- Puls, J. 1992. α -Glucuronidase in hydrolysis of wood xylans. In Visser, J., Beldman, G., Kusters-van Someren, M.A., and Voragen, A.G.J. (eds) *Xylans and xylanases*. Elsevier Science Publisher, Amsterdam. pp213-224.
- Puls, J. and Schuseil, J. 1993. Chemistry of hemicellulose structure and enzymes required for hydrolysis. In Coughlan, M.P., and Hazlewood, G.P. (eds). *Hemicellulose and Hemicellulases*. pp 1-27.
- Puls, J., Pountanen, K., and Lin, J.J. 1990. Enzymatic accessibility of hemicellulose in hardwood pulps to enzymes. In Kirk, T.K., and Chang, H.-M. (eds). *Biotechnology in pulp and paper manufacture. Applications and fundamental investigations. Proceedings of the fourth International Conference on Biotechnology in the Pulp and Paper Industry*. pp 183-190.
- Punt, J., Van Biezen, N., Conesa, A., Albers, A., Mangnus, J., and Van Den Hondel, C. 2002. Filamentous fungi as cell factories for heterologous protein production. *Trend. Biotechnol.* **20** (5):200-206.
- Ragauskas, A., Nagy, M., Kim, D.H., Eckert, C.A., Hallett, J., and Liotta, C.L. 2006. From wood to fuels- integrating biofuels and pulp production. *Industr. Biotechnol.* **2** (1):55-65.
- Rahman, A.K.M.S., Kato, K., Kawai, S., Takamizawa, K. 2003b. Substrate specificity of α -arabinofuranosidase from *Rhizomucor pusillus* HHT-1. *Carbohydr. Res.* **338**:1469-1476.
- Rahman, A.K.M.S., Kawamura, S., Hatsu, M., Hoq, M.M., and Takamizawa, K. 2001. Physicochemical properties of a novel α -L-arabinofuranosidase from *Rhizomucor pusillus* HHT-1. *Can. J. Microbiol.* **47**:767-772.

- Rahman, A.K.M.S., Sugitani, N., Hatsu, M., and Takamizawa, K. 2003a. A role of xylanase, α -L-arabinofuranosidase, and xylosidase in xylan degradation. *Can. J. Microbiol.* **49**:58-64.
- Ramírez, F., Puls, J., Zúñiga, V., and Saake, B. 2008. Sorption of corn cob and oatspelt arabinoxylan onto softwood pulp. *Holzforsch.* **62** (3):329-337
- Rattan, O., Izydorczyk, M. S., and Biliaderis, C.G. 1994. Structure and rheological behaviour of arabinoxylans from Canadian bread wheat flours. *Lebns. Wiss. Technol.* **27**:550-555.
- Reis, C.P., Neufeld, R.J., Rbeiro, A.J., and Veiga, F. 2006. Nanoencapsulation I. Methods for preparation of drug-loaded polymeric nanoparticles. *Nanomed., Nanotechnol. Biol. Medi.* **2**:2:8-21.
- Reis, D., Roland, J.C., Mosiniak, M., Darzens, D., and Vian, B. 1992. The sustained and warped helicoidal pattern of a xylan-cellulose composite: the stony endocarp model. *Protoplasma* **166** (1-2):22-34.
- Renard, C.M.G.C., and Jarvis, M.C. 1999. A cross-polarization, magic-angle-spinning, ^{13}C -nuclear-magnetic-resonance study of polysaccharides in sugar beet cell walls. *Plant Physiol.* **119**:1315-1322.
- Riley, G.L., Tucker, K.G., Paul, G.C., Thomas, C.R. 2000. Effect of biomass concentration and mycelial morphology on fermentation broth rheology. *Biotechnol. Bioeng.* **68** (2):160-172.
- Roche Applied Science. 2007. Working with proteins. The RAS lab FAQs. 3rd edition.
- Roger, R.D., and Seddon, K.R. 2003. Ionic liquids-solvents for the future. *Science* **302**:792-793.
- Rombouts, F.M., Voragen, A.G.J., Searle-van Leeuwen, M.F., Geraeds, C.C.J.M., Schols, H.A., and Pilnik, W. 1988. The arabinanases of *Aspergillus niger*-purification and characterization of two α -L-arabinofuranosidases and endo-1,5- α -L-arabinanase. *Carbohydr. Polym.* **9**: 25-47.
- Roos, A.A., Persson, T., Krawczyk, H., Zacchi, G., Ståhlbrand, H. 2009. Extraction of water-soluble hemicelluloses from barley husks. *Bioresour. Technol.* **100**:763-769.
- Rose, S.H., and Van Zyl, W.H. 2002. Constitutive expression of *Trichoderma reesei* β -1,4-xylanase gene (xyn2) and the β -1,4-endoglucanase gene (egl) in *Aspergillus niger* in molasses and defined glucose media. *Appl. Microbiol. Biotechnol.* **58**:461-468.
- Rose, S.H., and Van Zyl, W.H. 2008. Exploitation of *Aspergillus niger* for the heterologous production of cellulases and hemicellulases. *Biotechnol. J.* **2**:167-175.
- RSA-Logical Technologies (RSA-LT). 2003. Bamboo set to make its mark on SA building, paper sectors. <http://www.engineeringnews.co.za/article/bamboo-set-to-make-its-mark-on-sa-building-paper-sectors-2003-01-31> (accessed on 28th April, 2010).
- Ryabova, O., Vrsanská, M., Kaneko, S., Van Zyl, W.H., and Biely, P. 2009. A novel family of hemicellulolytic alpha-glucuronidase. *FEBS Lett.* **583** (9):1457-62.
- Saake, B., Busse, T., and Puls, J. 2005. The effect of xylan adsorption on the properties of sulphite and kraft pulps. In 59th Appita Conference and Exhibition incorporating the 13th International Symposium on Wood, Fibre and Pulping Chemistry. *Conference proceedings*:141-146. Auckland, Newzealand.(online

- <http://search.informit.com.au/browsePublication;isbn=0958554897;res=IELHSS>. (Accessed 12 February, 2010).
- Saha, B.C. 2000. α -L-Arabinofuranosidase: biochemistry, molecular biology and application in biotechnology. *Biotechnol. Advanc.* **18**:403-423.
- Saha, B.C., and Bothast, R.J. 1998. Purification and characterization of novel thermostable α -L-Arabinofuranosidase from color-variant strain of *Aureobasidium pullulans*. *Appl. Environ. Microbiol.* **64**:216-220.
- Saitô, H. 2005. Conformational and dynamic aspects of polysaccharide gels by high-resolution solid state NMR. In Dumitriu, S. (ed.) *Polysaccharides structural diversity and functional versatility*. 2nd edition. Marcel Dekker, New York, USA. pp 253-266.
- Saitô, H., Ando, I., and Naito, A. 2006. The solid state NMR spectroscopy for : *Principles and application*. Springer, The Netherlands. pp 464.
- Salmela, M., Alén, R., Vu, M.T.H. 2008. Description of Kraft cooking and oxygen-alkali delignification of bamboo by pulp and dissolving and dissolving material analysis. *Industrial crops and products*. **28**:47-55.
- Sambrook, J., Fritsch, E.F., and Maniatis, T. 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp1659.
- Sánchez-Torres, P., González-Candelas, L., Ramón. 1996. Expression in a wine yeast strain of the *Aspergillus niger* abfB gene. *FEMS Microbiol. Lett.* **145**:189-194.
- Sasaki, M., Adschiri, T., Arai, K. 2003. Fractionation of sugarcane bagasse by hydrothermal treatment. *Biores. Technol.* **86**:301-304
- Saxena, A., and Ragauskas, A. J. 2009. Water transmission barrier properties of biodegradable films based on cellulosic whiskers and xylan. *Carbohydr. Polym.* **78** (2):357-360.
- Saxena, A., Elder, T.J., Pan, S., and Ragauskas, A.J. 2009. Novel nanocellulosic xylan composite film. *Composites: Part B* **40**:727-730.
- Schönberg, C., Oksanen, T., Suurnäkki, A., Kettunen, H., and Buchert, J. 2001. The importance of xylan for the strength properties of spruce kraft pulp fibres. *Holzforsch.* **55**(6):639–44.
- Schooneveld-Bergamns, M.E.F., Beldman, G., and Voragen, A.G.J. 1999. Structural features of (Glucurono) arabinoxylans extracted from wheat bran by barium hydroxide. *J. Cereal Sc.* **29**:63-75.
- Schwikal, K., Heinze, T., Ebringerová, A., Petzold, K. 2006, Cationic xylan derivatives with high degree of functionalization. *Macromol. Symp.* **232**:49-56.
- Scurlock, J.M.O., Dayton, D.C. and Hames, B. 2000. Bamboo: An overlooked biomass resource? *ORNL/TM-1999/264*. Oak Ridge National Laboratory, Oak Ridge, Tennessee. pp34.
- Segel, I.H. 1976. Biochemical calculations. John Wiley & Son Inc. pp 441.
- Seri, K., Sanai, K., Matsuo, N., Kawakubo, K., Xue, C. Y., and Inoue, S. 1996. L-Arabinose selectively inhibits intestinal sucrase in an uncompetitive manner and reduces glycaemic response after sucrose ingestion in animals. *Metabolism* **45**:1368–1374.
- Shallom, D., and Shoham, Y. 2003. Microbial Hemicellulases. *Curr. Opin. Microbiol.* **6**:219-228.
- Shao, S., Wen, G., and Jin, Z. 2008. Changes in chemical characteristics of bamboo (*Phyllostachys pubescens*) components during steam explosion. *Wood Sci. Technol.* **42**:439-451.

- Shatalov, A. A., and Pereira, H. 2004. Uronic (hexenuronic) acid profile of ethanol-alkali delignification of giant reed *Arundo donax* L. *Cellulose*. **11**:109-117.
- Shatalov, A.A., Evtuguin, D.V., and Neto, C.P. 1999. (2-O- α -D-Galactopyranosyl-4-O-methyl- α -D-glucurono)-D-xylan from *Eucalyptus globulus* Labill. *Carbohydr. Res.* **320**:93-99.
- Shen, J., Song, Z., Qian, X., and Liu, W. 2009. Modification of papermaking grade fillers: A brief review. *Biores.* **4** (3):1190-1209.
- Shuler M.L., and Kargi F. 2002. Bioprocess engineering: Basic concepts. 2nd edition. Prentice Hall Upper Saddle River, NJ. pp479.
- Siika-aho, M., Buchert, J., Vuorinen, T., Teleman, A., Tenkanen, M., Bailey, M., and Viikari, L. 2004. Method and enzymatic preparation for treatment of cellulose pulps. Patent #:20040069426. <http://www.freepatentsonline.com/y2004/0069426.html>. (Accessed July, 2007).
- Siika-aho, M., Tenkanen, M., Buchert, J., Puls, J., and Viikari, L. 1994. An α -glucuronidase from *Trichoderma reesei* RUT C-30. *Enzyme Microb. Technol.* **16**:813-819.
- Silva, A.K.A., Da Silva, E.L., Oliveira, E.E., Ngashima, T. Soares, L.A.L., Medeiros, A.C., Araújo, J.H., Araújo, I.B., Carriço, A.S. and Egito, E.S.T. 2007. Synthesis and characterization of xylan-coated magnetite microparticles. *Int. J. Pharm.* **334**:42-47.
- Silva, S. S., Felipe, M.G.A., Silva, J.B.A., and Prata, A.M.R. 1998. Acid hydrolysis of *Eucalyptus grandis* chips for microbial production of xylitol. *Proces. Biochem.* **33** (1): 63-67.
- Simão, J.P.E., Egas, A.P.V., Baptista, C.M.S.G., Carvalho, M.G., and Castro, J.A.A.M. 2005. Evolution of methyglucuronic and hexenuronic acid contents of *Eucalyptus globulus* pulp during kraft delignification. *Ind. Eng. Chem. Res.* **44**:2990-2996.
- Sims, I.M., and Newman, R.H. 2006. Structural studies of acidic xylans exuded from leaves of the monocotyledonous plants *Phormium tenax* and *Phormium cookianum*. *Carbohydr. Polym.* **63**:379-384.
- Sjöström, E. 1993. Wood Chemistry: Fundamentals and applications. 2nd edition. Academic Press Limited, London, UK. pp 293.
- Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., Crocker, D., 2006. Determination of structural carbohydrates and lignin biomass. *Analytical Procedure (LAP)* 002,. NREL Biomass Program. National Bioenergy Center.
- Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., and Templeton, D. 2005. Determination of total solids in biomass. *Laboratory Analytical Procedure (LAP)*, version 2005. NREL Biomass Program. National Bioenergy Center.
- Spiridon, J., and Popa, V.I. 2005. Hemicellulose structure and properties. In Dumitriu, S. (ed). *Polysaccharides structural diversity and functional versatility*. Marcel Dekker New York. pp 475-489.
- Sternemalm, E., Höije, A., and Gatenholm, P. 2008. Effect of arabinose substitution on the material properties of arabinoxylan films. *Carbohydr. Res.* **343**:753-757.
- Sugar Milling Research Institute (SMRI) website: <http://www.smri.org/> accessed 25th November 2009.
- Sun, J.X., Sun, R.C., Sun, X.F., and Su, Y.Q. 2004b. Fractional and physico-chemical characterization of hemicelluloses from ultrasonic irradiated sugarcane bagasse. *Carbohydr. Res.* **339**:291-300.

- Sun, J.X., Sun, X.F. Zhao, H.S., and Sun, R.C. 2004c. Isolation and characterization of cellulose from sugarcane bagasse. *Polym. Degrad. Stability* **84**:331-337.
- Sun, J.X., Sun, X.F., Sun R.C., and Su, Y.Q. 2004a. Fractional extractional and structural characterization of sugarcane bagasse hemicelluloses. *Carbohydr. Polym.* **56**:195-204.
- Sun, R. C., Fang, J.M., and Tomkinson, J. 2000b. Characterisation and esterification of hemicellulose from Rye. *J. Agric. Food Chem.* **48**:1247-1252.
- Sun, R., Lawther, J.M., and Banks, W.B. 1996. Fractional and structural characterization of wheat straw hemicellulose. *Carbohydr. Poly.* **29**:325-331.
- Sun, R.C., Tomkinson, J., Wang, Y.X., and Xiao, B. 2000a. Physico-chemical and structural characterization of hemicelluloses from wheat straw by alkaline peroxide extraction. *Polym.* **41**:2647-2656.
- Sun, X.F., Xu, F., Zhao, H., Sun, R.C., Fowler, P., and Baird, M.S., 2005. Physico-chemical characterization of residual hemicellulose isolated with cyanamide-activated hydrogen peroxide from organosolv pre-treated wheat straw. *Biores. Technol.* **96**:1342-1349.
- Swift, R.J., Karandikar, A., Griffen, A.M., Punt, P.J., Van Den Hondel, C.A.M.J.J., Robson, G.D., Trinci, A.P.J., and Wiebe, M.G. 2000. The effect of organic nitrogen sources on recombinant glucoamylase production by *Aspergillus niger* in chemostat culture. *Fungal Genet. Biol.* **31**:125-133.
- Tagawa, K., and Kaji, A. 1988. α -L-arabinofuranosidase from *Aspergillus niger*. *Meth. Enzymol.* **160**:707-712.
- Tajana, E., Fiechter, A; Zimmermann, W. 1992. Purification and characterization of two α -l-arabinofuranosidases from *Streptomyces diastaticus*. *Appl. Environ. Microbiol.* **58**:1447-1450.
- Talabardon, M., and Yang, S. 2005. Production of GFP and glucoamylase by recombinant *Aspergillus niger*: Effects of fermentation conditions on fungal morphology and protein secretion. *Biotechnol. Prog.* **21**: 1389-1400.
- Talahashi, N., and Koshijima, T., 1988. Ester linkages between lignin and glucuronoxylan in a lignin-carbohydrate complex from beech (*Fagus crenata*) wood. *Wood Sci. Technol.* **22**(3):231-241.
- TAPPI, 2002-2003. TAPPI standards methods for measuring pentosans in wood and pulp (T223 cm-84).
- TAPPI, 2002-2003. Test Method for determining carbohydrate composition of extractive-free wood and wood pulp by gas chromatograph. (T249 cm-85).
- TAPPI, 2002-2003. Test Method for preparation of wood for chemical analysis. (T264 om-88).
- Teleman, A., Harjunpää, V., Tenkanen, M., Buchert, J., Hausalo, T., Drankenberg, T., and Vuorinen, T. 1995. 4-Deoxy- β -L-*threo*-hex-4-enopyranosyluronic acid attached to xylan in pine kraft pulp and pulping liquor by ^1H and ^{13}C NMR spectroscopy. *Carbohydr. Res.* **272**:55-71.
- Teleman, A., Lundqvist, J., Tjerneld, F., Stålbrand, H., Dahlman, O. 2000. Characterization of acetylated 4-*O*-methylglucuronoxylan isolated from aspen employing ^1H and ^{13}C NMR spectroscopy. *Carbohydr. Res.* **329**: 807-815.
- Teleman, A., Tenkanen, M., Jacobs A., and Dahlman, O. 2002. Characterization of *O*-acetyl-(4-*O*-methylglucurono)xylan isolated from birch and beech. *Carbohydr. Res.* **337**: 373-377.
- Tenkanen, M., and Siika-aho, M. 2000. An α -glucuronidase of *Schizophyllum commune* acting on polymeric xylan. *J. Biotechnol.* **78**:149-161.

- Tenkanen, M., Soovre, M., Heikkinen, S., Talja, R., Helen, H., and Hyvönen, L. 2007. Cereal arabinoxylans as raw material for biodegradable films. *Proceedings workshop on functionalisation and analysis of hemicellulose for sustainable advanced products*. March 19-20. Germany. Pp 25-26. <http://www.bfafh.de/aktuell/pdf/costE41-proc07.pdf> (accessed 1 March 2010).
- The Biomass Corporation. 2009. Bamboo project status in South Africa. <http://www.biomasscorp.com/southafrica.htm> (accessed 28 April 2010)
- Timell, T.E. 1967. Recent progress in the chemistry of wood hemicelluloses. *Wood Sci. Technol.* **1**:45-70.
- Tunc, M.S., and Van Heiningen, A.R.P. 2008b. Hemicellulose extraction of mixed southern hardwood with water at 150 °C: effect of time. *Ind. Eng. Chem. Res.* **47**:7031-7037.
- Tunc, M.S., and Van Heiningen, A.R.P., 2008a. Hydrothermal dissolution of mixed southern hardwood. *Holsforsch.* **62**:539-545.
- Vaghefi, F., and Savitzky, M.F. 2005. Microcapsule matrix microspheres, adsorption-enhancing pharmaceutical composition and methods. *US Patent 6849271*.
- Van Beilen, J.B., and Li, Z. 2002. Enzyme technology: an overview. *Curr. Opin. Biotechnol.* **13**:338-344.
- Van Den Hombergh, J.P.T.W., Van Der Vondervoort, P.J.I., Fraissinet-Tachet, L., and Visser, J. 1997. *Aspergillus* as host for heterologous protein production: the problem of proteases. *Trends Biotechnol.* **15**: 256-263.
- Van Der Klashorst, G.H. 1989. Utilization of soda bagasse hemicellulose as corrugated board adhesive. In Hemingway, R.W., Corner, A.H., and Branham, S.J.(eds) *Adhesives from renewable resources*. Americ. Chem. Soc. pp305-325.
- Van Heiningen, A. 2006. Converting a kraft pulp mill into an integrated forest biorefinery. *Pulp & paper Canada.* **107**(6):38-43.
- Van Suijdam, J.C., and Metz, B. 1981. Influence of engineering variables upon morphology of filamentous molds. *Biotechnol. Bioeng.* **23**:111-148.
- Van Tomme, S.R., Storm, G., and Hennik, W.E. 2008. *In situ* gelling hydrogels for pharmaceutical and biomedical applications. *Int J. Pharm.* **355** (1-2):1-18.
- Van Zyl, P.J., Moodley, V., Rose, S.H., Roth, R.L., and Van Zyl, W.H. 2009. Production of the *Aspergillus aculeatus* endo-1,4- β -mannanase in *A. niger*. *J. Ind. Microbiol. Biotechnol.* **36**:611-617.
- Vázquez, M.J., Alonso, J.L., Domínguez, H., and Parajó, J.C. 2000. Xylooligosaccharides: manufacture and applications. *Trend. Food Sci. Technol.* **11**:387-393.
- Vázquez, M.J., Garrote, G., Alonso, J.L., Domínguez, H., and Parajó, J.C. 2005. Refining of autohydrolysis liquors for manufacturing xylooligosaccharides: evaluation of operational strategies. *Biores. Technol.* **96**(8):889-896.
- Veen, P.V.D., Flipphi, M.J.A, Voragen, A.G.J., and Visser, J. 1991. Induction, purification and characterisation of arabinases produced by *Aspergillus niger*. *Arch. Microbiol.* **157** (1):23-28
- Verdoes, J.C., Punt, P.J., and Van Der Hondel, C.A.M.J.J. 1995. Molecular genetic improvement for the over production of fungal proteins by filamentous fungi. *Appl. Microbiol. Biotechnol.* **43**:195-205
- Vignon, M.R. and Gey, C. 1998. Isolation, ^1H and ^{13}C NMR studies of (4-XXX-methyl-D-glucurono)-D-xylans from luffa fruit fibres, jute bast fibres and mucilage of quince tree seeds. *Carbohydr. Res.* **307**:107-111.

- Viikari, L. 2003. Lignocellulose modifying enzymes for sustainable technologies. In Mansfield, S.D., and Saddler, J.N. Applications of enzymes to lignocellulosics. *ACS Symp. Ser.* **855**:30-44.
- Viikari, L., Tenkanen, M., Buchert, J., Rättö, M., Bailey, M., Sii-kaaho, M., and Linko, M. 1993. Hemicellulases for industrial applications. In Saddler, J.N. (ed). Bioconversion of forest and agricultural plant residues C.A.B International, CABI, Wallingford, UK. pp131-182.
- Vincken, J.-P., De Kezer, A., Beldman, G., and Voragen, A.G.J. 1995. Fractionation of xyloglucan fragments and their interaction with cellulose. *Plant Physiol.* **108**:1579-1585.
- Virkki, L. Johansson, L., Ylinen, M., Maunu, S., and Ekholm, P. 2005. Structural characterization of water-insoluble nonstarchy polysaccharides of oats and barley. *Carbohydr. Polym.* **59**:357-366.
- Voragen, A.G.J., Gruppen, H., Verbruggen, M.A., and Vietor, R.J. 1992. Characterization of cereal arabinoxylans. In Visser, J., Beldman, G., Kusters-Van Someren, M.A., and Voragen, A.G.J. (eds). *Xylans and xylanases*. Elsevier Science Publisher, Amsterdam. Pp 51-67.
- Vu, T.H.M., Alén, R., and Pakkanen, H. 2003. Delignification of bamboo (*Bambusa procera acher*). Part 2 . Characterisation of kraft black liquors from different cooking conditions. *Holzforsch.* **57**:619-626.
- Vu, T.H.M., Pakkanen, H., and Alén, R. 2004. Delignification of bamboo (*Bambusa procera acher*) Part 1. Kraft pulping and the subsequent oxygen delignification to pulp with a low kappa number. *Industrial Crops and Products.* **19**:49–57.
- Vuorinen, T., Fagerstrom, P., Buchert, J., Tenkanen, M., and Teleman, A. 1999. Selective hydrolysis of hexenuronic acid groups and its application in ECF and TCF bleaching of kraft pulps. *J. Pulp Paper Sci.* **25** (5):155-162.
- Walker, E.F. 1965. Effects of the uronic caboxyls on the sorption of 4-*O*-methylglucuronoarabinoxylans and their influence on papermaking properties of cellulose fibres. *Tappi* : **48** (5):298-303.
- Wan, J., Wang, Y., and Xiao, Q. 2010. Effects of hemicellulose fibre structure and recycling characteristics of eucalyptus pulp. *Biores. Technol.* **101**:4577-4583.
- Wang, L., Ridgway, D., Gu, T., Moo-Young, M. 2005. Bioprocessing strategies to improve heterologous protein production in filamentous fungal fermentations. *Biotechnol. Adv.* **23**:115-129.
- Wang, W., Liu, X., Xie,Y., Zhang, H., Yu, W., Xiong, Y., Xiong,Y., Xie, W., and Ma, X. 2006. Microencapsulation using polysaccharides for drug delivery and cell implantation. *J. Mater. Chem.* **16**:3252-3267.
- Westbye, P., Svanberg, C., and Gatenholm, P. 2006. The effect of molecular composition of xylan extracted from birch on its assembly onto bleached softwood pulp. *Holsforsch.* **60**:143-148.
- Whish-Wilson, P. and Maley, S. 2002. Preliminary assessment of product & market opportunities for the bamboo industry in Far North Queensland. prepared for the Bio-Industry Cluster Far North Queensland .Bamtek and Kleinhardt FGI Pty Ltd. pp 66.
- Whitney, S.E.C., Brigham, J.E., Darke, A.H., Reld, J.S.G., and Gidley, M.J. 1995. *In vitro* assembly of cellulose/xyloglucan networks: ultrastructural and molecular aspects. *Plant J.* **8** (4):491-504.
- Wiebe, M. 2003. Stable production of recombinant proteins in filamentous fungi: problems and improvements. *Mycol.* **17** (3):140-144.

- Wiebe, M.G., Karandikar, A., Robson, G.D., Trinci, A.P., Candia, J.L., Trappe, S., Wallis, G., Rinas, U., Derkx, P.M., Madrid, S.M., Sisniega, H., Faus, I., Montijn, R., van den Hondel, C.A.M.J.J., and Punt, P.J. 2001. Production of tissue plasminogen activator (t-PA) in *Aspergillus niger*. *Biotechnol. Bioeng.* **76**:164–174.
- Wilkie, K.C.B. 1979. The hemicelluloses of grasses and cereals. . In: Tipson, R.S., and Horton, R. (eds.). *Adv. Carbohydr. Chem. Biochem.* **36**:215-264.
- Wilkie, K.C.B., and Woo, S.-L. 1977. A heteroxylan and hemicellulosic materials from bamboo leaves and a reconsideration of the general nature of commonly occurring xylans and other hemicelluloses. *Carbohydr.* **57**:145-162.
- Winter, H., Barakat, A., Cathala, B. and Saake, B. 2006. Preparation of arabinoxylan and its sorption on bacterial cellulose during cultivation. *Macromol. Symp.* **232**:74-84.
- Wong, K.K.Y., and Saddler, J.N. 1993. Application of hemicelluloses in the food, feed, and pulp and paper industries. In: Coughlan, P., and Hazlewood, G.P. (eds.). *Hemicellulose and Hemicellulases*. Portland Press. London. pp 127-143.
- Wood, T. M., and Macrae, S.I. 1996. Arabinoxylan –degrading enzymes systems of the fungus *Aspergillus awamori*: purification and properties of an α -L-arabinofuranosidase. *App. Microbiol. Biotechnol.* **45** (4):538-545.
- Xu, F., Sun, J.X., Liu, C.F., and Sun, R.C. 2006. Comparative study of alkali- and acidic organic solvent-soluble hemicellulosic polysaccharides from sugarcane bagasse. *Carbohydr. Res.* **341**:253-261.
- Xu, J., Wang, L, Ridgway, D., Gu T., and Moo-Young, M. 2000. Increased heterologous protein production in *Aspergillus niger* fermentation through extracellular protease inhibition by pellet growth. *Biotechnol. Prog.* **16**: 222-227.
- Yan, Z., Liu, Q., Deng, Y., and Ragauskas, A. 2005. Improvement of paper strength with starch modified clay. *J. Appl Polym. Sci.* **97**:44-50.
- Yang, R., Xu, S., Wang, Z., and Yang, W. 2005. Aqueous extraction of corn cob xylan and production of xylooligosaccharides. *LWT* **38**:677-682.
- Yang, R., Zhang, C., Feng, H., and Yang, W. 2006. A kinetic study of xylan solubility and degradation during corncob steaming. *Biosys. Engin.* **93** (4): 375-382.
- Yi, Y., Neufeld, R.J., and Poncelet, D. 2005. Immobilization of cells in polysaccharide gels. In: Dumitriu, S. (ed). *Polysaccharides structural diversity and functional versatility*. Marcel Dekker New York. pp 867-891.
- Yoon, S-Y. 2007. Bonding material coated clay for improving paper properties. *PhD Thesis*. School of Chemical and Biomolecular Engineering, Georgia Institute of Technology. Pp 197.
- Yoshida, S., Kuno, A., Saito, N., Aoyama, M., and Kusakabe, I. 1998. Structure of xylan from culms of bamboo grass (*Sasa senanensis* Rehd.). *J. Wood Sci.* **44** (6):457-462.
- Yu, Q., Zhuang, X., Yuan, Z., Wang, Q., Qi, W., Wang, W., Zhang, Y., Xu, J., and Xu, H. 2010. Two-step liquid hot water treatment of *Eucalyptus grandis* to enhance sugar recovery and enzymatic digestibility of cellulose. *Biores. Technol.* **101** (13): 4895-4899.
- Zaide, G., Shallom, D., Shulami, S., Zolotnisky, G., Golan, G., Baasov, T., Shoham G., and Shoham, Y. 2001. Biochemical Characterization and Identification of Catalytic residues in α -glucuronidase from *Bacillus stearothermophilus* T-6. *Europ. J. Biochem. FEBS.* **268**:3006-3016.

- Zhang, Y.H.P. 2008. Reviving the carbohydrate economy via multi-product lignocelluloses biorefineries. *J. Ind. Microbiol. Biotechnol.* **35**:367-375.
- Zhou, Q., Baumann, M.J., Piispanen, P.S., Teeri, T.T., and Brumer, H. 2006. Xyloglucan and xyloglucan endo-transglycosylases (XET): Tools for *ex vivo* cellulose surface modification. *Biocatal. Biotransform.* **24**:107-120.